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Award Number: DAMD17-99-1-9550

TITLE: Liposome Delivery of a Potential Antidote for Mustard Exposure

Subtitle: An Overview and integration of data for phase one experiments by the Mustard Consortium, regarding the use of antioxidants as a counter measure to mustard

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REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Annual (1 Oct 99 - 30 Sep 00)

4. TITLE AND SUBTITLE

Liposome Delivery of a Potential Antidote for Mustard Exposure

Subtitle: An Overview and integration of data for phase one experiments by the Mustard Consortium, regarding the use of antioxidants as a counter measure to mustard

5. FUNDING NUMBERS

DAMD17-99-1-9550

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Report contains color graphics.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

See report

14. SUBJECT TERMS

15. NUMBER OF PAGES
106

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

The mechanism of action of mustards has been unknown since its initial development. To date there has been no counter measure to this vesicant that could be utilized for systemic and skin exposure (*both of which occur in combat conditions*). The members of the Mustard Consortium hypothesized that oxidative reactions were a significant part of the inflammatory process that is responsible for the damage that occurs when an animal is exposed to mustards. If indeed oxidative reactions were a significant part of the mechanism of action, then the use of antioxidants would decrease damage to the exposed tissue. We also hypothesized that a new technology, the antioxidant liposome could be used as vehicle for the delivery of antioxidants. Antioxidants have been demonstrated to have a significant protective effect for both prior, and post exposure to mustards. The Consortium's strength is in the multidisciplinary approach to this complex problem. The group is able to incorporate information from outside sources and generate new information at faster rate, due to the ability to consult other members involved in different aspects of the same research project. Multiple models have been developed which also rapidly increase useful experimental data in the determination of a counter measure.

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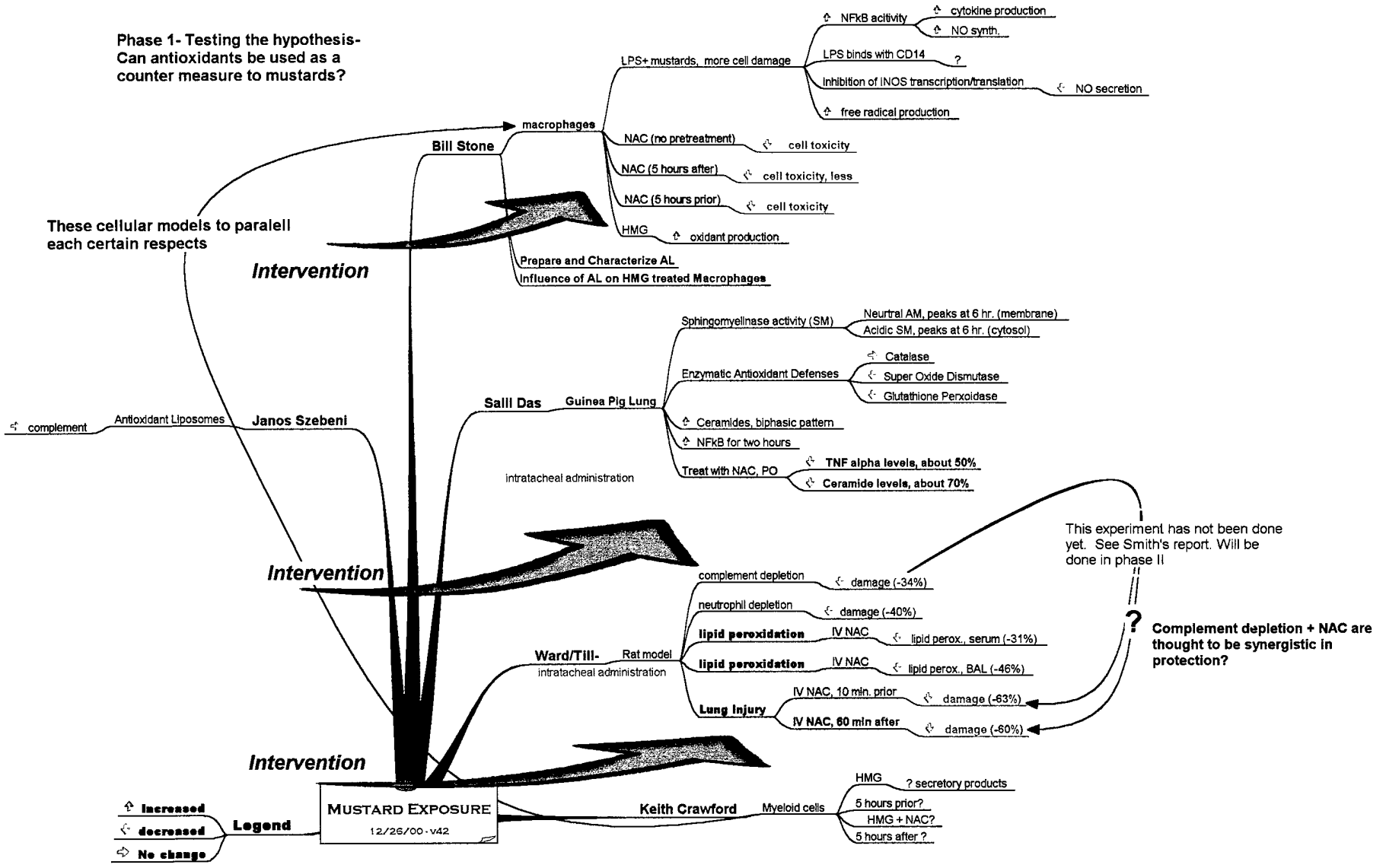
Individual Investigator Reports

Introduction

The Mustard Consortium (consisting of six investigators) used a multidisciplinary approach to the problem of attempting to develop counter measures to mustards. The Consortium has found that NAC (N-acetyl cystiene) a glutathione precursor, was able to ameliorate much of the damage that was induced by the mustard analogue (HMG or 2-chloroethyl ethyl sulfide) in both in vitro and in vivo models. NAC was able to achieve a high level of protection of cellular viability, decrease TNF alpha, decrease ceramide (an indicator of apoptosis) levels, and decrease lipid peroxides. NAC showed a high level of protection whether it was administered prophylactically, simultaneously with HMG, or after the addition of HMG. NAC is an excellent counter measure in these early studies in the rat, guinea pig and the macrophage cell models. HMG inhibited nitric oxide production the macrophage cellular model. The antioxidant liposome (AL) containing glutathione and vitamin E had no effect on complement activity.

NAC is a safe, inexpensive, and readily available compound. In this first year of research we have used NAC as the free drug. In future studies we will encapsulate the NAC and other antioxidants in liposomes. We believe that the AL when used in the other limbs of the study will have an enhanced salutary effect, in comparison to NAC as the free drug. The liposome encapsulated antioxidant formulation may well serve as the foundation for an eventual antidote to mustards; as well

Overview of Research Progress for the Mustard Consortium- The First Year



Summation of Laboratory Results (see the graphical representation of the overview; for details see the individual reports)

University of Michigan- Peter Ward and Gerd Till
HMG administered via the intratracheal route to the lung of the rat (refer to section A)

- Lipid peroxides were generated in the plasma and the bronchial lavage fluid
- Neutrophil depletion of the animal lessened the damage to the lungs
- Depletion of circulating complement lessened the lung damage
- NAC (N –acetyl cystiene) administered after the lung was exposed to mustard was protective
- NAC (N –acetyl cystiene) administered prior to the lung being exposed to mustard was also protective

Discussion of results:

The intratracheal instillation of HMG is thought to deliver more drug to the deeper parts of the lung, as opposed to an aerosol delivery. Lipid peroxides were generated within the lung, but were not isolated to the lung. Lipid peroxides were also found in the plasma. This is similar to the "second hit" phenomenon in severe burns. Wherein a burn injury to the skin, results in ARDS (adult respiratory distress syndrome). ARDS also occurs in mustard gas exposure. The generation of lipid peroxides within the confines of the lung, as well as spreading to distant sites has the potential to not only damage the lung tissue it self, but other tissues as well. Circulating lipid peroxides also could lead to wide spread activation of monocytes and neutrophils (a systemic inflammation).

Removal of neutrophils from circulation was also protective (40%). Neutrophils generate free radicals, and contain enzymes that are cell toxic. Their removal and subsequent protection demonstrates how one component of the acute inflammatory reaction contributes to tissue damage induced by mustards.

Circulating complement, another component of acute inflammation, was depleted. The protection achieved was 34%. Another way to interpret this evidence that depletion of complement is protective, inhibition of complement would also be protective. It is therefore reasonable to believe that complement inhibitors would be a useful adjunct to the treatment of mustard gas exposure.

NAC is a precursor, for cysteine. Cysteine is converted to GSH (glutathione) in macrophages. Glutathione is one of the major intracellular water soluble antioxidants. Both cysteine and GSH are antioxidants, they both contain thiol groups that are able to quench free radicals. **The intravenous administration of NAC resulted in a diminution of lipid peroxides in the bronchial lavage fluid (decrease by 31%) as well as in the plasma (decreased by 46%).** Therefore the lipid peroxides were being quenched in two different compartments: the plasma and the interior surfaces of the lung.

NAC administered prophylactically, 10 minutes prior to the administration of HMG afforded protection to lung tissue (approximately 63%).

NAC administered 60 minutes after the exposure to HMG was also protective to lung tissue. Both of these findings are clinically relevant. The implication is that being able to pretreat a soldier could afford a high level of protection. This method of prophylactic treatment is particularly important, since it is unknown when a soldier will be exposed to mustards. At the time of an attack, there is likely insufficient time to don protective gear if the soldier is near the point of detonation of the shell or down wind from the blast. Having an antioxidant formulation already in the blood of the soldier, could afford him or her the additional minutes that are needed to don the protective gear and survive the attack. **NAC administered one hour <u>after the exposure</u> to HMG also exhibited excellent protection of lung tissue (60%).** This is also clinically relevant. If this information was extrapolated to the battlefield, it could represent the situation of an individual who is exposed to mustards but has had treatment delayed for approximately one hour. If the NAC was then administered reasonable protection could be afforded to the individual. This is a particularly valuable limb of the experiment. If NAC were clinically proven to be efficacious, this would be the most commonplace treatment modality. In a massive mustard attack, whether it is in the battlefield theater, or in the civilian population there would be many casualties that would be need to be treated. In all likelihood, very few if any would have been pretreated.

Meharry Medical College- Salil Das

Intratracheal instillation of HMG in the guinea pig (refer to section B)

- The guinea pig lung is very similar to the human lung
- TNF alpha levels are elevated in the first one hour then decline rapidly over a six hour period
- Both acid and neutral sphingomyelinases are elevated with consequent ceramide levels increased. Ceramide levels remained elevated in the untreated guinea pigs for 7-14 days.
- Superoxide dismutase and glutathione peroxidase (two of the three enzymes that defend against free radicals) showed initially increased activity (1-6 hours) but continued to decrease from day 1-21.
- Catalase (the third of three enzymes that defend against free radicals) activity was essentially unchanged
- In this limb of the experiment NAC was given by mouth for three days prior to the instillation of HMG.
- NFkB was initially increased and then disappeared
- **NAC *decreased* TNF alpha by 45-50%**
- **NAC decreased ceramide levels by 70%**

Discussion of Results

TNF alpha is known to mediate apoptosis and also induces free radicals. And was elevated by the administration of HMG. Free radicals activate the sphingomyelinases with the release of ceramides. The ceramides are a secondary messenger, and its presence is indicative of apoptosis.

Superoxide and glutathione peroxidase activity were initially increased. It is reasonable to assume that superoxide as well as lipid peroxides were being generated. It is likely that these enzymes were being modulated by the HMG. It is possible that the free radical burden became larger over time?

NFkB, the transcription protein, was found to have increased activity in response to HMG. This increased in activity is indicative of an increase in the production of inflammatory cytokine production.

In this limb of the study has demonstrated through biochemical markers, that the prophylactic use of oral the oral administration of NAC was protective. In this case it was protective in decreasing one index of apoptosis, the ceramides. NAC also decreased TNF alpha, which significantly reduced a mediator of apoptosis and free radicals.

East Tennessee State

Preparation of Liposomes (refer to section C)

- Small unilamellar liposomes were produced containing fat soluble vitamin E (both gamma and alpha- tocopherol were used)
- Characterization of the antioxidant liposomes are on going, novel methods are being develop to fulfill this limb of the experiment.

Discussion of Results

These newly devised methods for characterization are likely to be patentable.

East Tennessee State

RAW267.7 Macrophages (M)- cell model (refer to section C)

- LPS from gram-negative bacteria make M more susceptible to the effects of HMG; binds with CD 14; activates NFkB and consequently activates the transcription of NO synthetase
- HMG decreases NO production, at higher levels it is completely suppressed
- HMG inhibits iNOS transcription/translation
- HMG with out LPS generated free radicals. HMG with LPS show a greater amount of free radical production.
- **NAC added to the cell culture five hours prior to the addition of HMG showed approximately 90% protection**
- **NAC added five hours after HMG showed approximately a 75% level of protection.**
- **NAC added to cell cultures simultaneously with HMG, demonstrated greater than 90% protection**

Discussion of Results

- In theses studies it was seen that HMG inhibited iNOS. This resulted in decreased production of NO. If the same iNOS inhibition occurs in vivo, it could have devastating effects on a mammal. NO in humans contribute to: arterial dilatation, bronchodilitation, antioxidant activity, inhibition of apoptosis, etc. NAC did not protect iNOS.
  If similar studies in vivo demonstrate iNOS to be inhibited after HMG exposure, then a method of protecting iNOS should be developed. This is an area that deserves more study.
  NAC was added to the culture in three different timed sequences: prophylactically, simultaneously and after the addition of HMG. In all three timed sequences NAC was protective at high levels.

Center for Blood Research

Human Dendritc Cell-Monocyte Model (refer to section D)

- Dendritic cells, when isolated produce higher levels of complement, chemokines and cytokines
- Dendritic cells in the presence of monocytes produce higher levels of C1inhibitor
- Dendritic cells or monocytes isolated, produce higher levels of C1; although dendritic cells> monocytes production of C1 inhibitor

Discussion

It is known that circulating monocytes release a higher level of chemokine, cytokines and complement, in comparison to residential monocytes cells. It may be the case that plasma lipid peroxides activate monocytes consequently generating free radicals and releasing complement.

If mustards activate dendritic cells in the skin then they would significantly contribute to the damage by the release of complement.

A combination of the AL with complement inhibition may neutralize the effects of HMG activated monocytes and dendritic cells.

Body

A singular approach to a problem can result in a myopic solution. The design of the Mustard Consortium was done with the goal in mind to meld different scientific disciplines and coordinate activities in their approach to the difficult problem of developing a counter measure to mustards. The multidisciplinary approach creates an environment for the cross pollination of ideas. This readily does not occur on an ongoing basis with such a large group. Usually investigators stay within their own disciplines for collaboration, and do not derive the benefit of approaches that are outside of their discipline.

The Consortium configuration was developed in an effort to decrease the cycle of time that is usually found between the concept, execution, and the determination of an idea as a commercial product. The consortium consists of six investigators: Sail Das (biochemistry-Meharry Medical College), Bill Stone (biochemistry /liposomology-East Tennessee State), Keith Crawford (immunology-Center for Blood Research), Carl Alving (complement/liposomology-Walter Reed Army Institute of Biomembrane Research), Peter Ward (ARDS/Pathology-University of Michigan) and Milton G. Smith (inventor-Amaox, Ltd.)/Emergency Medicine). This newly formed network of scientist forms a "collective brain" that is able to share information in the gathering and in the dissemination of information (which is multidisciplinary) at a greater rate, due to continuous collaboration. The technology that is central to the work of the Consortium is the antioxidant liposome (AL; patent pending). The AL is thought to have broad application for diseases that have significant oxidative reactions, and can be used in both the military and civilian populations.

Since the discovery of mustards in the late 1800's, their mechanism of action has been unknown. The primary organs affected are the lung, eyes and skin. Mustard gas (1,1' thiobis [chloroethane]) a vesicant and radiomimetic does not have any known antidote since its synthesis over one hundred years ago. Mustards are radiomimetics, its damage has the characteristics of radiation exposure. It is well established that radiation damage is mediated by the generation of reactive oxygen intermediates (ROI). Free radicals or ROI have been shown to occur in mustard exposure and inflammation.

The acute inflammatory process is a complex cascade of events; one subset that has been under appreciated is the role of oxidants. Free radicals or oxidants upregulate NFkB, a transcription protein, that produces a number of inflammatory cytokines (e.g., TNF alpha, GMSCF, and Il 1-beta). Oxidants and cytokines form a positive feed back loop with NFkB that results in an amplification of the inflammatory process, and the induction of apoptosis. The amplification of the inflammatory process consequently causes tissue destruction. Antioxidants down regulate NFkB activity, reduce inflammatory cytokines and are anti-inflammatory. We hypothesized that mustards or its analogue induced tissue destruction by inflammation, and that the generation of a free radicals was a significant subset of the inflammatory process. It was also hypothesized that targeted delivery of antioxidants could ameliorate tissue damage produced by mustards..

Three models were successfully established: macrophage, guinea pig lung and rat lung models. Oxidative reactions were present in all three of the models. In the rat and guinea pig model the intratracheal route was used to administer HMG. The intratracheal delivery of HMG, would emulate an individual who was close to the point of a blast (almost ground zero), the worst -case scenario. In contrast the aerosol model likely represents inhalation of mustards at site distant from ground zero. And would actually represent the *best-case scenario* for emulation of the inhalation of mustards. The transcription protein, NFkB, translates for the inflammatory cytokines (TNF alpha, GMCSF, Il 1-beta). In the guinea pig model NFkB levels were elevated, but then disappeared after two hours.. Oxidants are a stimulus for NFkB activity. Antioxidants have the opposite effect, in that they down regulate the activity of NFkB.

NAC (N-acetyl cystiene) is a cystiene donor that is converted to glutathione intracellularly. In the cellular model, prophylactic use of the NAC five hours prior to the use of the HMG conferred approximately 85%-90% protection (maintenance of cellular viability). A similar result was found in the rat model, although at a level of approximately 60% protection was achieved. Therefore the two parallel, but independent, studies confirm these findings of pretreatment (or prophylaxis) using NAC as being protective.

In the guinea pig model TNF alpha and ceramide levels were both decreased. One source of TNF alpha is the production by the transcription factor NFkB, by inducible genes. The exhibited *decrease* in TNF alpha, in the guinea pig, could be explained by the increase in the redox potential of macrophages, and consequent decrease in activity of NFkB. Ceramide levels decreased approximately 70%, indicative of a diminution of apoptosis.

Thus far it has been unreported in any animal model that prophylactic administration of a drug was protective at the levels shown in the experiments by the Consortium (intravenous NAC 63%; in the cell line-approximately 90%). The cell model and rat model were independent of each other, but show a consistent result in NAC affording tissue protection. This finding sets the groundwork to establish a prophylactic medication to be used as a counter measure. Prophylactic use of a counter measure has the obvious benefits of lessening the damage of mustards when it is encountered. This is clearly advantageous, since the onset of an attack is unknown. Respiratory and skin protective gear may be rendered ineffective if the mustard overcomes the individual. The prophylactic medication could markedly increase the amount of time between the exposure and the onset of debilitating symptoms; possibly providing sufficient time to don protective wear.

NAC was protective when there was a simultaneous administration with HMG in the cellular model. The clinical corollary is for acute treatment to be instituted immediately after exposure to the mustard. Although this exact experiment was not done in the animal models, it is reasonable to extrapolate that a similar result of protection would occur.

Post exposure treatment was nearly as effective as pre-exposure treatment in the rat model. The post exposure use of NAC simulates the conditions wherein an individual has been exposed to mustards, but acute treatment was delayed. This may be the case in a "hot zone" where immediate evacuation is impossible. NAC could then be given when evacuation to another site is accomplishment. Post exposure treatment was effective in the rat and in the cell model. In the rat model NAC was administered at 60 minutes, in cell model at 300 minutes (five hours) after exposure to HMG. Protection in the rat model was 60%, whereas in the cell model it was about 75%.

Aerosol administration of NAC was not tired in this round or experiments. Aerosol delivery would be an additional route of delivery that can be used concomitantly with the other routes that have been utilized our studies. It would seem plausible that the aerosol route of delivery used in addition to the oral, or intravenous routes of delivery would be synergistic. It is anticipated that the aerosol treatment and intravenous administration would be the most desirable method of delivery of antioxidants for the inhalation of mustards. In battle field conditions inhalation therapy would easily utilized as a stabilizing treatment. The aerosol could be continued used with intravenous therapy.

In the rat model complement depletion demonstrated protection from injury at 34%. This limb of the experiment demonstrates the potential contribution of complement to the overall damage elicited by HMG. NAC was not used in conjunction with the depletion of complement in the phase I of experimentation.
It would be anticipated that NAC used in conjunction with complement inhibition, would give a higher level of protection, as compared to the use of NAC alone (which was about 60%). No complement inhibitors were used in this phase of the experiments.

The use of the antioxidant precursor NAC has been clearly demonstrated to be effective by two different routes of administration in the animal models (oral and intravenous). The level of protection in the cell model achieved was higher than that of the animal models; this is due to a direct application of NAC to the cells that have been exposed to HMG. That would be in contrast to the animal models wherein there would be a dilutional effect of the NAC at the target site where the pathologic oxidative reactions are occurring. It is believed that a similar level of protection achieved in the cell model could be achieved the animal models, if the level of the antioxidant (or precursor) was sufficient at the target site of the oxidative reactions. We have hypothesized that this dilutional effect could be largely over come by the use of liposomes.

Our second phase of experimentation will be to continue to characterize, develop, and use the liposome encapsulated antioxidants (AL) in the different models.

Liposomes offer the advantage of a higher level of drug delivery in comparison to use of the free drug (in our example it would be NAC and other antioxidants); as well as being timed release in order to extend the therapeutic window. These AL could be administered intravenously, topically, or by aerosol. AL administered by aerosol has been demonstrated to have a high therapeutic efficacy with a low dose and greater tissue retention (Fan, J.; et. al.: Surgery 2000; 128:332-8).

It is anticipated that optimal protection can be achieved in the animal models with the use of both aerosol and intravenous administration of the AL. This dual method of delivery would be expected to be superior to intravenous, intratracheal or aerosol delivery of the free drug. (Although there may be a role using both free NAC as well as liposome encapsulated NAC). Oxidative reactions in biological systems create free radicals that that occurs in the aqueous and lipid compartments of cells. NAC would be distributed in the water soluble compartments, but would not quench free radicals in the lipid soluble compartments. Lipid soluble antioxidants (e.g., the tocopherols or beta-carotene) are required to quench lipid peroxides. The lipid soluble antioxidants may be useful in reducing oxidative reactions in humans. They can only be administered intravenously if first solublized in liposomes.

Based on this early data, it would appear that a theoretically optimal treatment might be the use of the AL (antioxidant liposome) that was also able to inhibit complement. This would allow the delivered antioxidants to quench free radical reactions, have an anti-inflammatory effect, and neutralize circulating complement. If the complement depletion studies and the intravenous administered NAC are synergistic, protection in animals could well go beyond 70%. Further funding would be needed to develop this counter measure.

Problems Encountered

The funding for Keith Crawford was received in August 2000. There was a significant delay due to regulatory hurdles that had to be overcome prior to the release of the funds.

Key Research Accomplishments

Rat Lung Model (University of Michigan)

- Development of a rat model for intratracheal administration of mustard
- Mustards generate free radicals in both plasma and bronchial alveolar lavage fluid
- Neutrophil depletion of the animal decreases lung damage
- Complement depletion of the animal decreases lung damage
- NAC (N-acetyl cystiene) protects from lung injury **after** exposure to mustards (60%)
- " " " " " prior to exposure to mustards (63%)

Guinea Pig Model (Meharry Medical College)

- Development of the intratracheal administration of mustard
- Two of the three enzymatic antioxidant defenses are decreased (superoxide dismutase and glutathione peroxidase), there was no significant change in the catalase levels
- Ceramides increase in a biphasic pattern
- NFkB activity is increased for two hours
- NAC decreased tumor necrosis factor alpha by 50%
- NAC decreased ceramide levels by 70%

Macrophage Cell Line Model (East Tennessee State)

- Development of macrophage cell model
- NAC used either at the time of application of the mustard, prior to mustards, or after mustards **was protective to the cells**
- NFkB activity is increased
- Cytokine production increased
- Nitric oxide secretion is decreased, but nitric oxide synthetase is increased
- Free radicals are generated in cells exposed to mustards

Human Immune Cell Model (Center for Blood Research)- In progress

Antioxidant Liposome Interaction With Complement (Walter Reed Army Institute of Biomembrane Research)

- The antioxidant liposome has no significant effect on complement activity

Reportable Outcomes

1. A conference was held in Crystal City Virginia on October 20th, 2000. In attendance were the members of the Consortium. Also in attendance were members of the Army and Capital Hill. See document from the conference. In this conference strategy for the next phase of research was discussed, as well as research techniques and data that was presented.

2. Animal models developed:
   - Intratracheal administration of HMG in the Rat lung model (the same model is used for ARDS)
   - Intratracheal administration of HMG in the Guinea Pig lung model

3. Cell Model developed:
   - Macrophage cellular model

4. Liposome Interaction with complement- no effect

Conclusions

In vivo and in vitro models were developed; all have shown consistent and corresponding results in the significant protective effect that NAC (a water soluble antioxidant) has against the effects of HMG. NAC was protective when given by direct addition to cell culture, oral route or intravenously (in animals). **It has been demonstrated that three phases of treatment are possible:**

1) ***prophylaxis,***
2) ***acute treatment (at the time of exposure)***
3) ***post exposure treatment.***

HMG inhibits nitric oxide (NO) production in the cellular model. If NO is inhibited in animal models, then serious consideration should be given to protecting the production of nitric oxide synthase.
Excellent success was achieved in what we believe is a worst-case scenario, the intratracheal delivery of HMG. A perfected AL would be expected to have an enhanced half life of protection, as well as enhanced drug delivery to target sites. A liposome encapsulated antioxidant formulation would represent a readily achievable goal as counter measure for mustard exposure. The AL as a counter measure would likely have an extremely low complication rate. In these early studies NAC was used, it is well regarded as safe and is currently used clinically in acetaminophen (Tylenol) toxicity. Liposomes are already used commercially for drug delivery. Mustards are known to produce ARDS its victims. There is no clinically available drug that is being used to treat ARDS, a common complication of trauma. *The AL that will be developed as a counter measure for mustards could be used for many others disease, such as ARDS, heart attacks, and strokes, etc. Currently ARDS has a* ***fifty percent mortality rate***. AL in animal models has already been shown to be efficacious in the ARDS animal model.

Areas of expansion of research efforts that would require additional funding are:

- Aerosol delivery of AL, which has already shown efficacy in the ARDS animal model (similar damage is seen in mustard lung damage) should be developed for use as a counter measure in future experiments. Aerosol delivery can be included in future experiments with additional funding.
- Elucidating the role of complement on the systemic and pulmonary response to the exposure of HMG.
- Utilization of Knock out mice (complement inactivated); AL liposomes that inactivate complement and the development of a new complement inhibitory protein could be developed.
- Development of an agent to protect NO production.

References:

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Appendix:
Hand out from the Crystal City Meeting October 20th, 2000.
Peter Ward document Section A

Section A

Peter Ward and Gerd Till

The analog of mustard gas, 2-chloroethyl ethyl sulfide ($Cl\text{-}CH_2CH_2SCH_2CH_3$, "half mustard gas," HMG), when instilled into the airways of rats, induced acute lung injury characterized by extensive intrapulmonary hemorrhage and edema. These effects were dependent on the dose of HMG and proportional to the time after exposure to HMG. For example, at 4 hours after the intrapulmonary deposition of 6.4 mg HMG per kg body weight, lung injury, as determined by the extravasation of radiolabeled bovine serum albumin (^{125}I-BSA), increased seven-fold above background values. Interventional studies, employing cobra venom factor for complement depletion or antibodies against rat neutrophils, showed that the development of HMG-induced lung injury was dependent on complement and the availability of neutrophils, respectively. Because the appearance of lipid peroxidation products in plasma of HMG-treated animals suggested involvement of toxic oxygen metabolites, N-acetyl-L-cysteine (NAC), a well-known anti-oxidant, was administered intravenously and was shown to have powerful, protective effects related to the dose administered and the time of its administration. NAC administration could be withheld for one hour after exposure of lung to HMG and still confer high degrees of protection. Antioxidants such as catalase, dimethyl sulfoxide or dimethyl thiourea were effective only when given prior to HMG instillation.

(4) INTRODUCTION

Our understanding of the pathomechanisms of sulfur mustard (mustard gas, CCl-CH2-CH2-S-CH2-CH2-Cl)-induced cell and tissue injury is still very limited. Based on our long-standing experience with experimental pulmonary injury models in the rat, we decided to use this animal species to study the effects of half-mustard gas (2-chloroethylethyl sulfide, Cl-CH2-CH2-S-CH2-CH3) on lung tissues. During initial in vivo experiments, we would expose the alveolar spaces of rats to varying concentrations of half-mustard gas (HMG) and determined the extent of lung injury by monitoring the extravasation of ^{125}Iodine-labeled bovine serum albumin (^{125}I-BSA) into alveolar spaces at various time points (1 to 4 hours) post intrapulmonary application of HMG. Once we had established HMG-induced lung injury in the rat, systematic studies would ensue to define the roles of inflammatory cells (neutrophils, alveolar macrophages) and mediators (oxidants, cytokines, chemokines, C5a). We believe that these studies will significantly further our understanding of the mechanisms by which half-mustard gas can cause cell and tissue injury in the lungs.

(5) BODY

Initial work focused on the development of a rat model of HMG-induced acute lung injury. We used male Long Evans rats, 300-350 grams body weight, for the half mustard gas (HMG) lung injury studies. This decision was based on our long-standing experience with this strain of rats in our other models of acute lung injury (1-3). Using these rats for our studies, anesthesia is performed by intraperitoneal injections of ketamine hydrochloride, 100 mg/kg. The rats are kept under anesthesia until sacrifice at the end of the experiment.

Lung injury is determined by the extravasation of radiolabeled protein into alveolar spaces. Therefore, experimental rats receive an intravenous injection of 1.5 μCi ^{125}I-BSA/kg at 10 minutes prior to HMG application. At the end of the experiment (at 4 hours post HMG, unless otherwise stated), 1 ml of blood is drawn from the posterior vena cava, and the thorax cut open to rinse the lung vasculature (via pulmonary artery) with 10 ml of phosphate-buffered saline (PBS). Using a gamma counter, the radioactivity (cpm) in the whole lung and in 1 ml of blood is

determined. Lung injury values are calculated by dividing total lung cpm by cpm present in 1 ml of blood.

Initial attempts to induce lung injury by depositing HMG in the lower parts of the rat trachea failed. Most of the rats died within minutes of HMG application due to asphyxia. Only when we deposited HMG deep into the right or left lung via a P52 catheter, the rats survived and developed lung injury. Another important finding was that HMG needed to be dissolved in ethanol first before addition to phosphate-buffered saline. We have established the following optimal concentrations for the induction of HMG-induced acute (4-hour) lung injury in he rat: Intrapulmonary Injection (per kg body weight) of 6 µl (6.42 mg) 2-chloroethyl ethyl sulfide (half-mustard gas, HMG) dissolved in 174 µl ethanol and 1020 µl phosphate-buffered saline (PBS).

As can be seen in Figure 1 (see Appendix), intrapulmonary injection into rats of HMG resulted in the development of dose-dependent acute lung injury, as determined at four hours post HMG application. Maximum 4-hour injury was achieved by injecting a dose of 9 µl(9.63 mg) HMG per kg body weight. In order to be able to monitor any decreases or increase in lung injury following various interventional procedures, we chose a HMG dose of 6 µl(6.42 mg) per kg body weight as a standard dose causing an injury of 1.72 injury values. Having established a "working dose" of HMG in our lung injury rat model, we used this dose to determine the time dependency of HMG-induced lung injury in the rat. The results of these studies are summarized in Figure 2 (see Appendix). Measuring at 0, 2, 4, and 6 hours post HMG injection, a clear time dependency of HMG-induced lung injury could be observed. For practical reasons (medium grade injury, reasonable time), we decided to chose the 4 hour time point as a standard for our model. In summary, our rat model of HMG-induced acute lung injury is characterized by the following parameters: HMG dose of 6.42 mg/kg given intrapulmonary dissolved in ethanol and PBS, lung injury determined at 4 hours post HMG injection by the extravasation of ^{125}I-BSA(bovine serum albumin). Under these conditions (see Figure 3, Appendix), HMG-dependent lung injury values increase about sevenfold above background values of ethanol/PBS.

Having established a rat model of HMG-induced lung injury, we started a series of experiments to determine whether blood neutrophils and/or the complement system play a role in the pathogenesis of HMG-induced lung injury. To deplete rats of their blood neutrophils,

commercially available rabbit antiserum against rat neutrophils was injected intraperitoneally (1 ml/rat) at 24 hours prior to HMG injection. Hemocytometer readings were done to determine the blood neutrophil counts prior to the injection of HMG. A blood neutrophil count of less than 250 per cmm (more than 90% reduction of normal) was considered satisfactorily. Complement depletion of rats was performed as reported previously (1). In short, cobra venom factor, purified from cobra venom, was injected intraperitoneally at concentrations of 20 units per rat on days 1 and 2. On day 3, blood samples were taken and the serum tested for complement activity by performing a CH50 assay (lysis of antibody-sensitized sheep red blood cells). Successful complement depletion resulted in zero CH50 values. The results of these studies are summarized in Figure 4 (see Appendix). As can be seen, both, depletion of complement and neutrophils resulted in a reduction of HMG-induced lung injury by 34% and 40%, respectively. The observations suggest that complement activation products as well as blood neutrophils are participating in the full development of HMG-induced lung injury.

Since inflammatory neutrophils are known to produce toxic oxygen metabolites, and because recent evidence pointed towards involvement of oxidants in mustard gas-induced blistering of skin (4), we examined serum and bronchoalveolar lavage (BAL) fluids of HMG-treated rats for the presence of lipid peroxidation products as evidence of oxidant production. Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (5). In this series of studies, we also included rats that were treated with the antioxidant, N-acetyl-L-cysteine prior to injection of HMG. The results are depicted in Figures 5 and 6 (Appendix). Serum as well as BAL fluids of HMG-treated rats showed significant increases in thiobarbituric acid reactive substances (TBARS). These increases in lipid peroxidation products could be significantly reduced (by 31% in serum and by 40% in BAL) when experimental animals were pretreated with NAC, which was given intravenously at a dose of 20 mg/kg, 10 minutes prior to HMG treatment.

We now asked the question, whether antioxidants could also attenuate HMG-induced lung injury. To answer this question, we used a variety of different antioxidants in addition to NAC. We will first discuss the results obtained with non-NAC antioxidants. The data are shown in Figure 7 (Appendix). To our surprise, only pretreatment with the hydroxyl radical scavengers,

dimethyl thiourea (DMTU, 1.0 g/kg, i.p.) and dimethyl sulfoxide (DMSO, 1.5 ml/kg, i.p.) resulted in protection from HMG-induced lung injury by 34% and 55%, respectively. The other antioxidants, including resveratrol, the iron chelators, desferal and 2,3-dihydroxybenzoic acid, or a mixture of superoxide dismutase and catalase, had no significant effect on the development of HMG-induced lung injury. In these experiments, we use antioxidant concentrations that had been shown to be effective in our other rat models of oxidant-induced acute lung injury (1-3).

When NAC was employed to study its effect on HMG-induced lung injury, quite dramatic effects were observed. When various concentrations of NAC were injected intravenously at 10 minutes prior to HMG treatment, a dose-dependent protection from lung injury could be observed (Figure 8, Appendix) reaching a maximum protection of 63 percent, when a NAC dose of 20 mg/kg was injected (Figure 9, Appendix). Most importantly, when the optimal NAC dose of 20 mg/kg was injected into rats at various time points before or after intratracheal injection of HMG, significant protection from lung injury could be observed even when NAC treatment occurred as late as 60 minutes post HMG treatment (see Figure 10, Appendix). These observations suggest that oxidants are involved in the pathogenesis of HMG-induced lung injury and that NAC might be a useful antidote to HMG poisoning.

(6) KEY RESEARCH ACCOMPLISHMENTS

- Established an animal (rat) model of half-mustard gas(HMG)-induced acute lung injury
- Demonstration that both neutrophils and complement are involved in the pathogenesis of HMG-induced lung injury
- Demonstration of role for oxidants in HMG-induced acute lung injury
- Established N-acetyl-L-cysteine is a potent antidote against HMG-induced lung injury

(7) REPORTABLE OUTCOMES

* An Abstract (see Appendix) has been submitted to the forthcoming meeting of Experimental Biology in Orlando, FL, March 31 – April 4, 2001
* An animal model has been developed (as described above)

(8) CONCLUSIONS

Our observations suggest that the development of half-mustard gas induced lung injury is dependent on the availability of both neutrophils and complement, and is largely mediated by toxic oxygen metabolites.

The latter is supported by our findings that N-acetyl-L-cysteine, a well-known anti-oxidant, has powerful, protective effects related to the dose administered and the time of its administration.

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(10) APPENDICES:

* Figures 1 - 10
- Abstract (submitted to forthcoming meeting in Orlando, FL) see below

- **Abstract submitted to Experimental Biology 2001, Orlando, FL, March 31-April 4, 2001`**

Protection From Half-Mustard Gas-Induced Acute Lung Injury in the Rat

McClintock, S.D., Till, G.O., and Ward, P.A.

Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109.

There is accumulating evidence that exposure to sulfur mustard gas results in a variety of tissue-damaging outcomes, especially in skin and lung, although our understanding of the mechanisms responsible for this injury is quite limited. We now have shown that an analog of mustard gas, 2-chloroethyl ethyl sulfide ($Cl\text{-}CH_2CH_2SCH_2CH_3$, "half mustard gas," HMG), when instilled into the airways of rats, induces acute lung injury characterized by extensive intrapulmonary hemorrhage and edema. These effects were dependent on the dose of HMG and proportional to the time after exposure to HMG. For example, at 4 hours after the intrapulmonary deposition of 6.4 mg HMG per kg body weight, lung injury values, as determined by the extravasation of radiolabeled bovine serum albumin (^{125}I-BSA), increased seven-fold above background values. Interventional studies, employing cobra venom factor for complement depletion or antibodies against rat neutrophils, showed that the development of HMG-induced lung injury was dependent on complement and the availability of neutrophils, respectively. Because the appearance of lipid peroxidation products in plasma of HMG-treated animals suggested involvement of toxic oxygen metabolites, N-acetyl-L-cysteine (NAC), a well-known anti-oxidant, was administered intravenously and was shown to have powerful, protective effects related to the dose administered and the time of its administration. NAC administration could be withheld for one hour after exposure of lung to HMG and still confer high degrees of protection. In contrast with these observations, antioxidants such as catalase, dimethyl sulfoxide or dimethyl thiourea were effective only when given prior to HMG instillation. Our data confirm that the rat lung is highly susceptible to HMG-induced injury and that the pulmonary damage is substantially reduced by treatment with NAC. (Supported by USAMRMC).

DOSE DEPENDENCY OF HALF-MUSTARD GAS (HMG)-INDUCED LUNG INJURY IN THE RAT

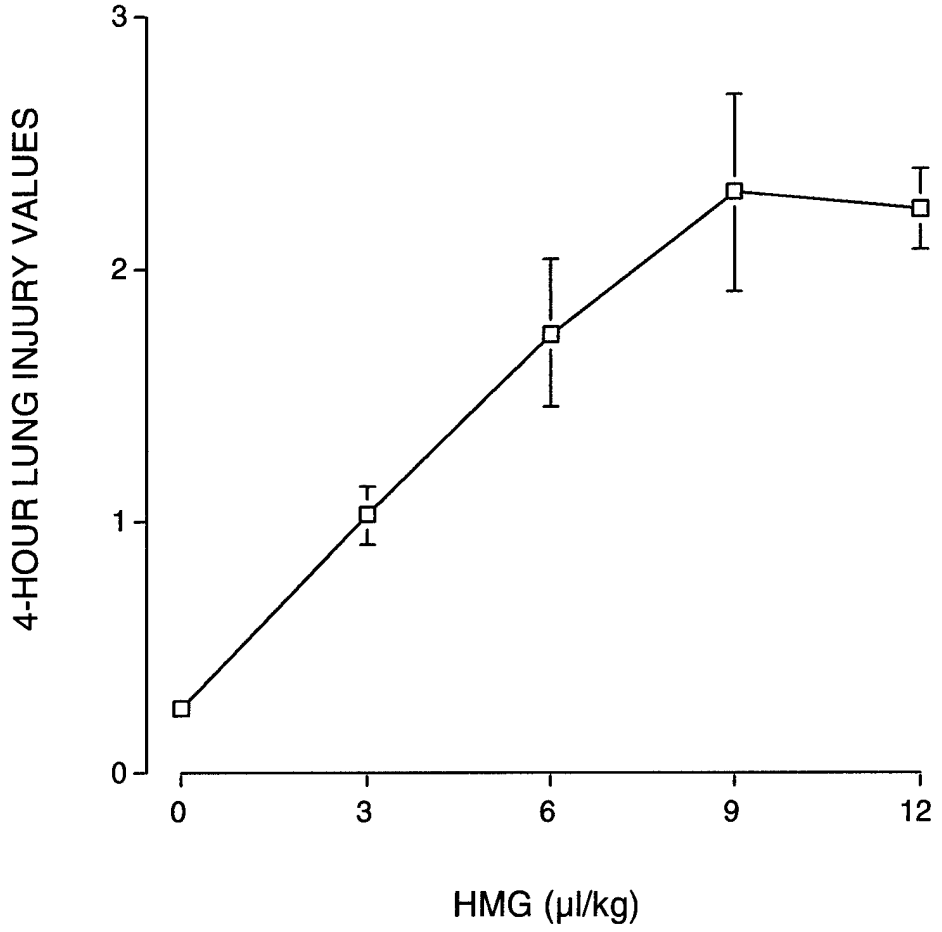

Figure 1 (Ward and Till, annual report)

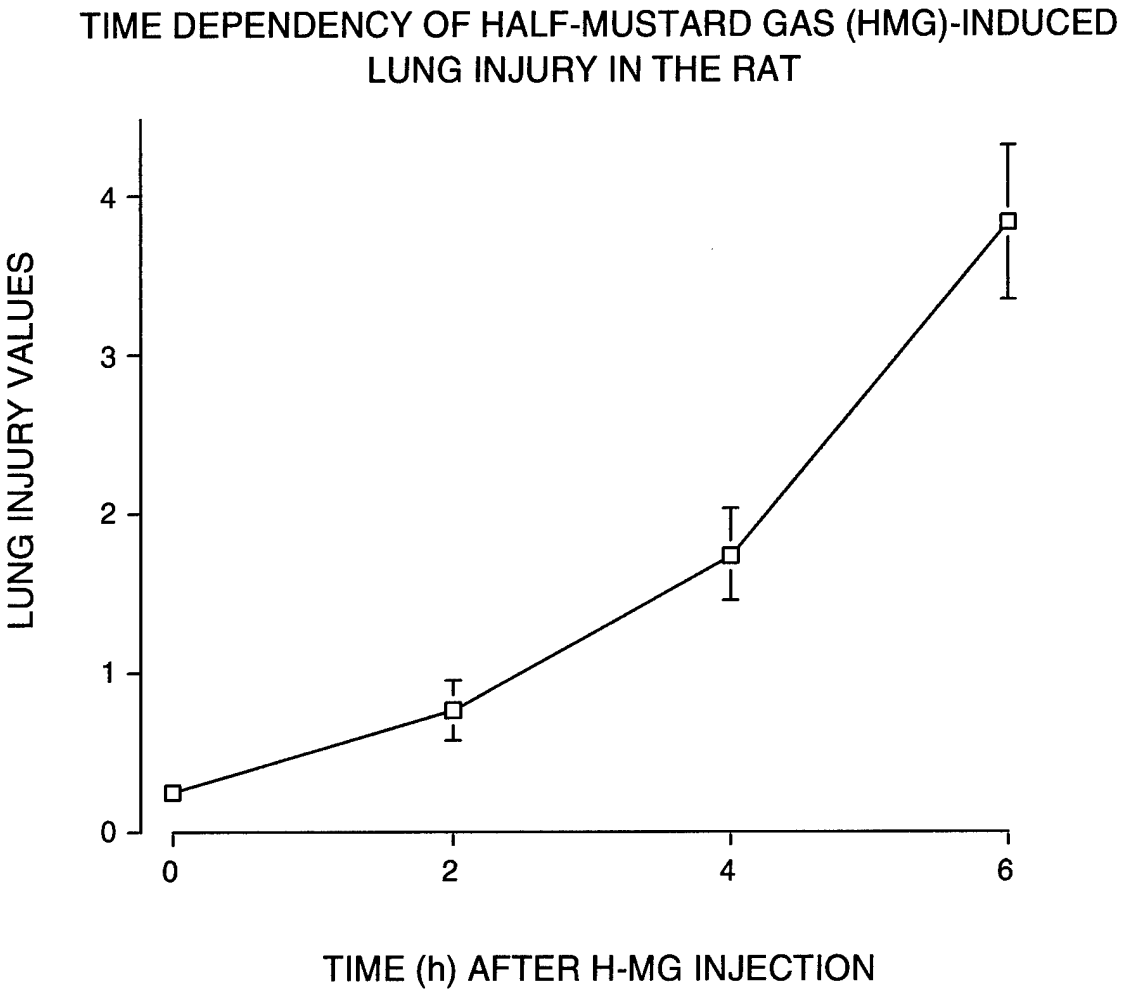

Figure 2 (Ward and Till, annual report)

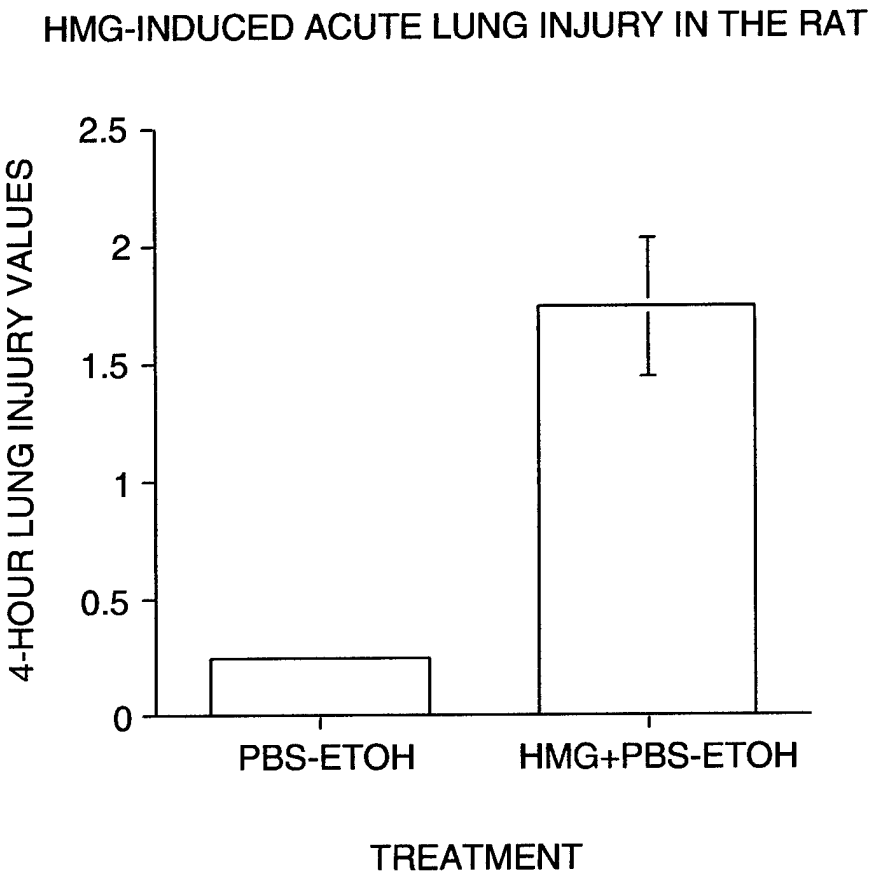

Figure 3 (Ward and Till, annual report)

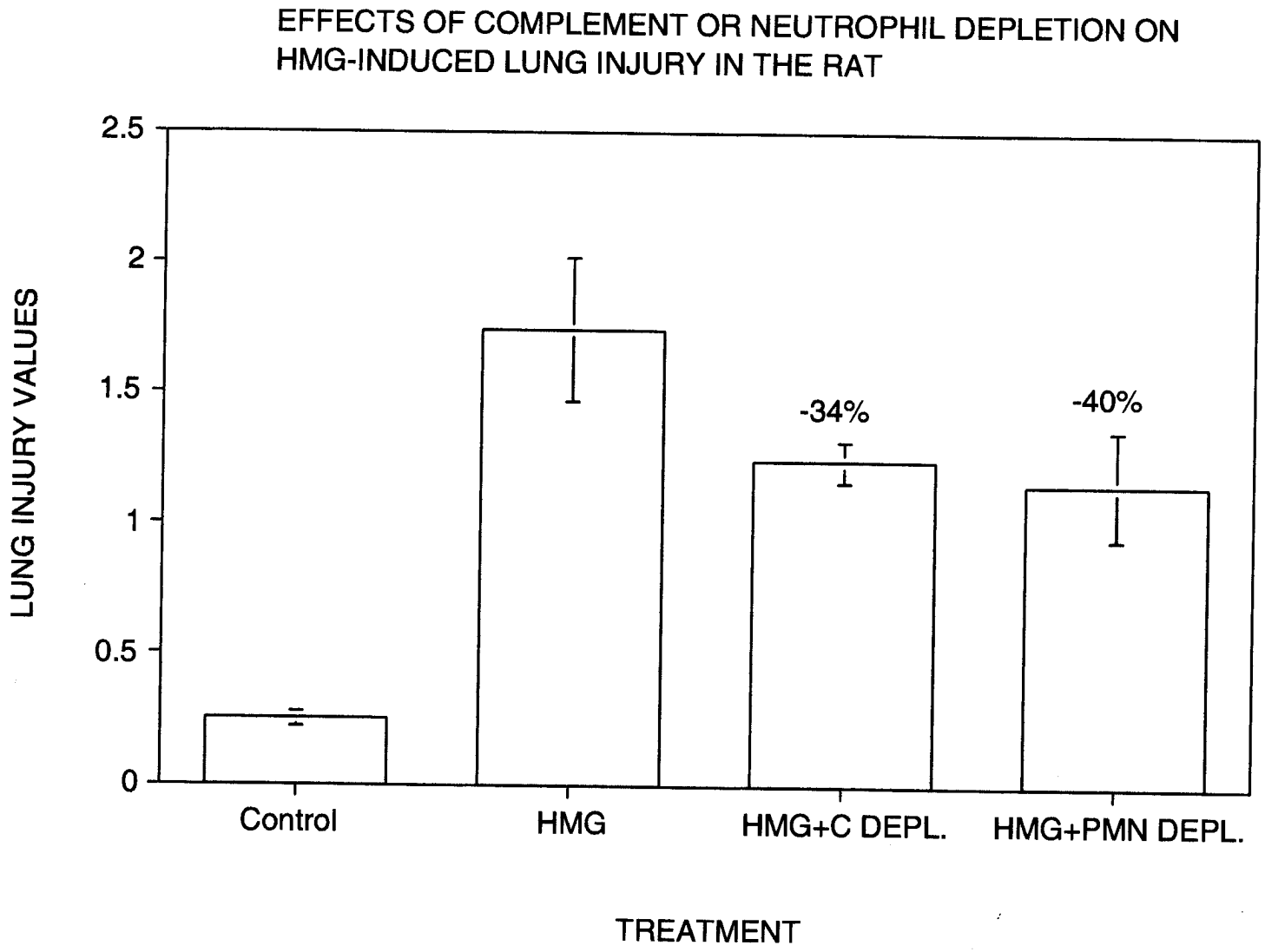

Figure 4 (Ward and Till, annual report)

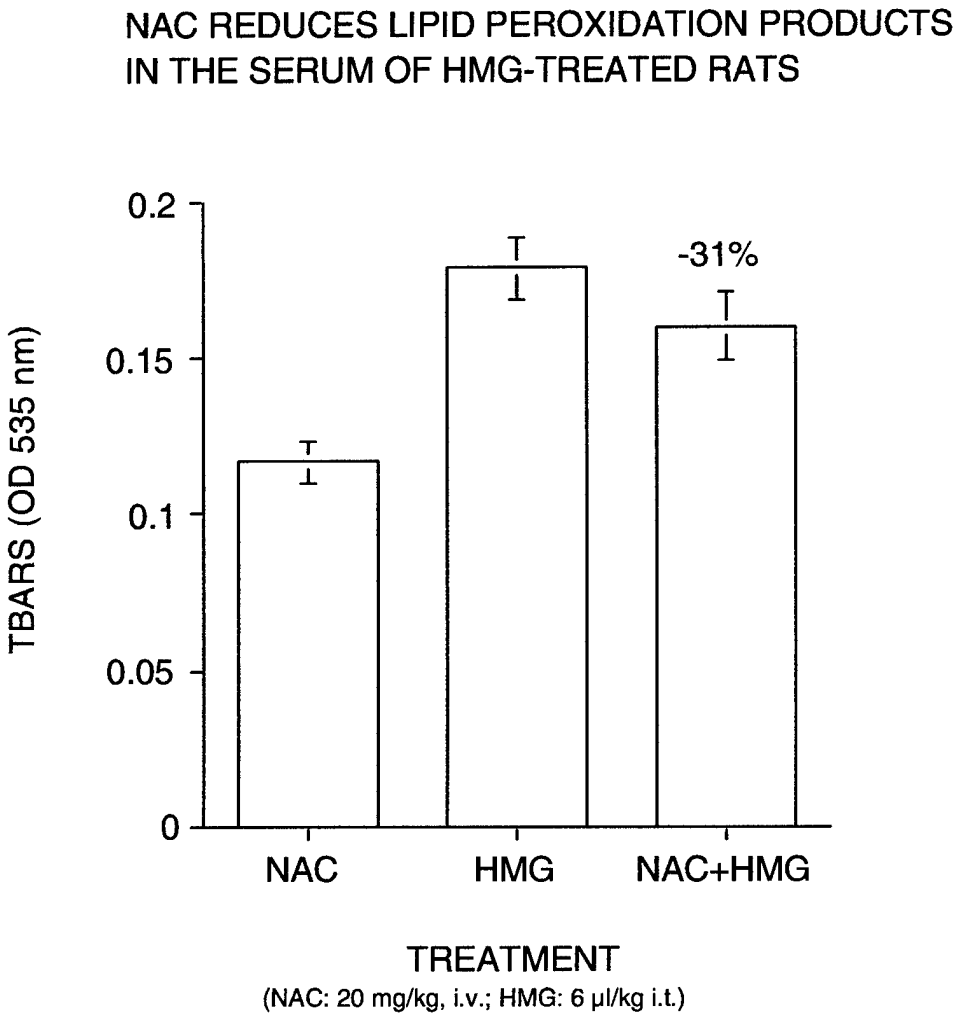

Figure 5 (Ward and Till, annual report)

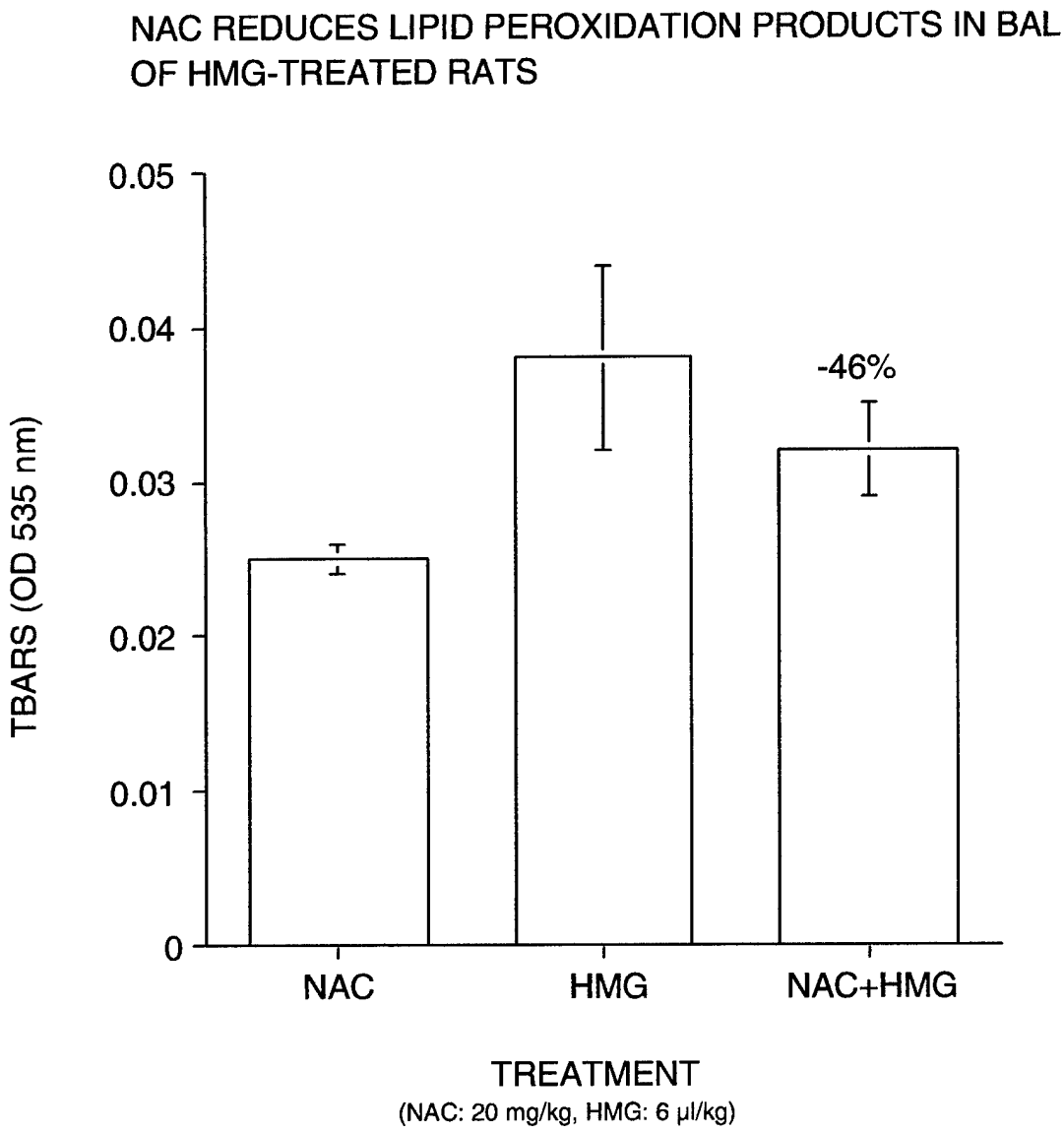

Figure 6 (Ward and Till, annual report)

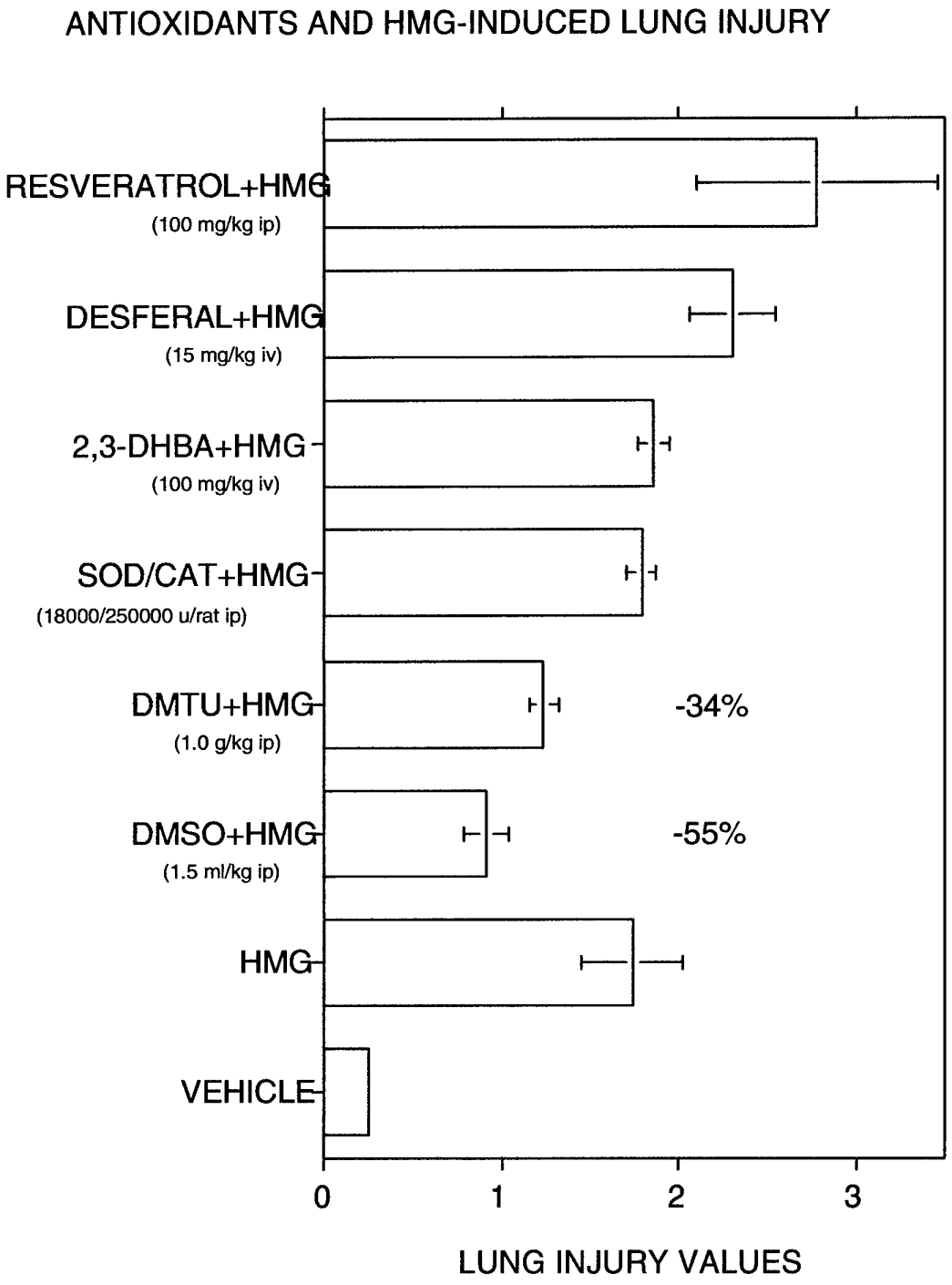

Figure 7 (Ward and Till, annual report)

Section B

Salil Das

The exact mechanism by which mustard gas exposure causes lung injury including ARDS is not well known. Since human volunteers can not be used for this type of study, we have developed a guinea pig model. Our initial studies have shown that guinea pigs exposed to chloroethyl ethyl sulfide (CEES) intratracheally, accumulate high levels of TNF-alpha, followed by activation of both acidic and neutral sphingomyelinases resulting in high accumulation of ceramides, a second messenger involved in cell apoptosis. This signal transduction event was associated with alteration in oxygen defense system and accumulation of free radicals. These biochemical changes were associated with lung damage, including edema, congestion, hemorrhage and inflammation and exclusion of type II cells into alveolar space. It is important to note that there was an activation of NFkB for a short period (1-2 hours after exposure); however, NFkB rapidly disappeared after 2 hours. It is possible that the initial activation was due to an adaptive response to protect the cells from damage since it is known that NFkB is an inhibitor of TNF-alpha/ceramide induced cell apoptosis. Since NFkB disappeared after 2 hours, the cells continued being damaged due to accumulation of ceramides. N-acetyl cysteine, an antioxidant caused 50% inhibition in the mustard gas induced signal transduction events. Therefore, N-acetylcysteine may be a potential antidote for mustard gas induced lung injury.

Table of Contents

INTRODUCTION:

Sulfur mustard has been used as a vesicant chemical warfare agent. Inhalation of mustard gas causes hemorrhagic inflammation to the tracheobronchial tree with severe pulmonary complications including Adult Respiratory Distress Syndrome (ARDS) (1). Most deaths are due to secondary respiratory infections. Several studies in rats and mice have shown that the mechanism of mustard gas action on lung, skin or other organs includes DNA alkylation, cross linking of DNA (2), activation of proteases (3), free radicals production (2), inflammations (4) and activation of tumor necrosis factor (TNFα), a part of the inflammatory cytokine cascade (5). Among the numerous mechanism proposed for the mustard gas toxicity, it appears that the initiation of TNFα cascade is the major pathway in the mustard gas mediated ARDS. Our ultimate goal is to develop drugs to counteract the mustard gas induced lung injury.

Since human volunteers cannot be used for such studies, we need to develop a good animal model for the study of mustard gas toxicity. We have established earlier that structurally and functionally, guinea pig lungs are more alike to human lungs in comparison to other animal species. Therefore, we are studying mustard gas mediated lung injury in guinea pigs to understand the mechanism of actions of mustard gas. It will be helpful for us to design drugs to prevent mustard gas induced lung injury after getting the clear picture of the mustard gas action on guinea pig lung.

WORK DONE DURING THIS PERIOD:

We have carried out our research according to our proposed specific aims in the approved statement of work. The results are described bellow:

Specific aim 1: To determine whether mustard gas exposure to guinea pigs causes an induction of TNFα in lung macrophages.

We have given single intratracheal injection (0.5 mg/kg body weight) of sulfur mustard gas (chloroethyl ethyl sulfide, CEES) to guinea pigs. At different time points, guinea pigs were sacrificed and lung was removed after perfusion. The lung was lavaged and TNFα concentrations were measured in lung lavage fluid, lung lavage macrophages and in lung tissue. The results indicated that the level of TNFα in lavage fluid was very low whereas a high level of TNFα accumulated in lung as well as in lavage macrophage within 1 hour of CEES exposure. The level of TNFα decreased rapidly after 1 hour and came to the normal level within 24 hours of CEES exposure (Fig 1).

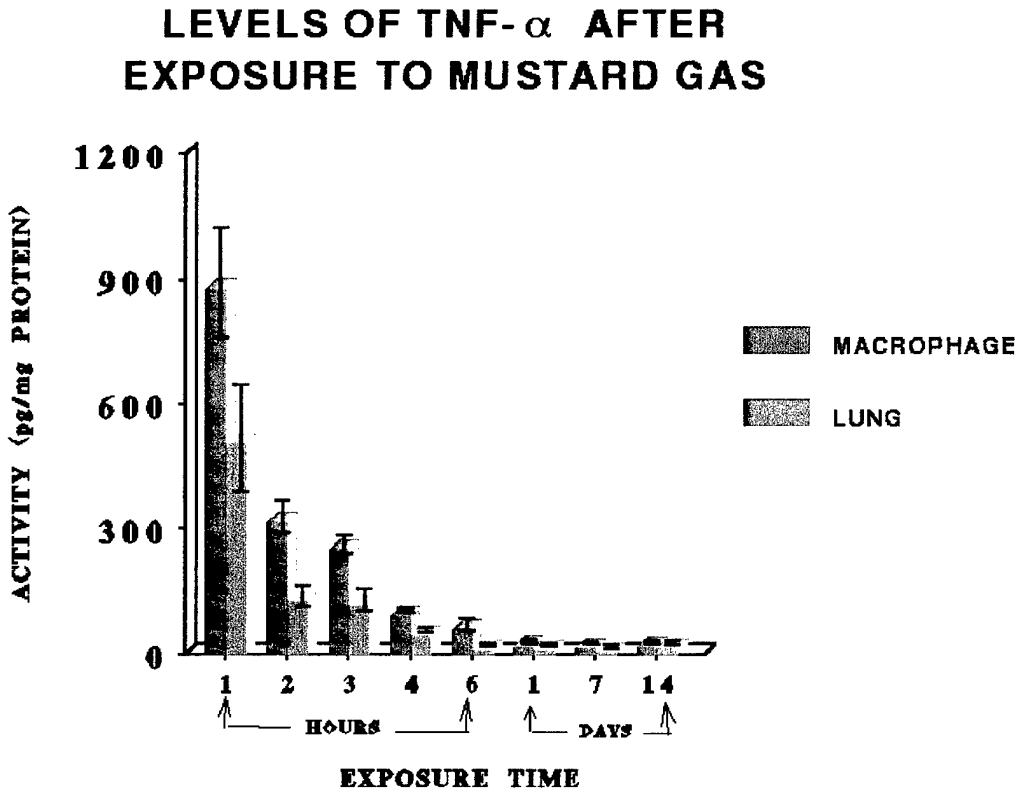

FIG. 1

Specific aim 2: To determine whether CEES exposure to guinea pigs causes accumulation of free radicals in lung macrophages and Type II cells:

Very short-term exposure (1 hr) caused an increase in the activities of both glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) and a decrease in the activity of catalase (Figs. 2-5). The activities of both SOD and GSH-Px started to decrease gradually reaching basal level at 6h, and continued to fall below the basal level at 7 days until 21 days. However, the catalase activity started to increase after two hrs reaching basal level at 4h, and after 24 hour started to decrease gradually below the basal level until 21 days. Thus, the lung damage due to mustard gas exposure may be associated with modulation of oxygen defense system of lung.

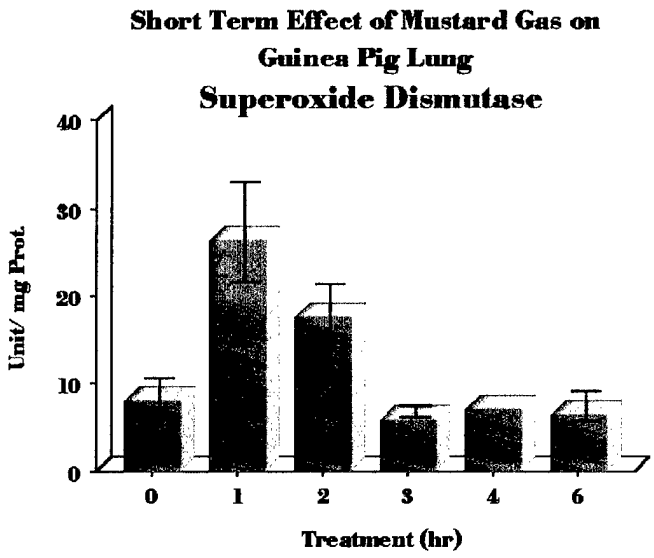

FIG. 2

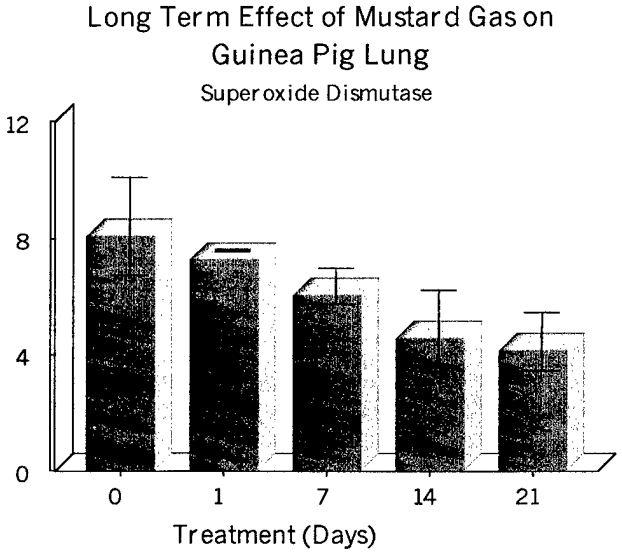

FIG. 3

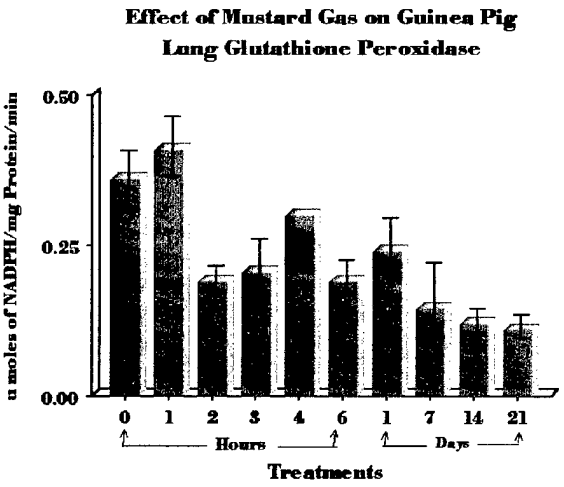

FIG. 4

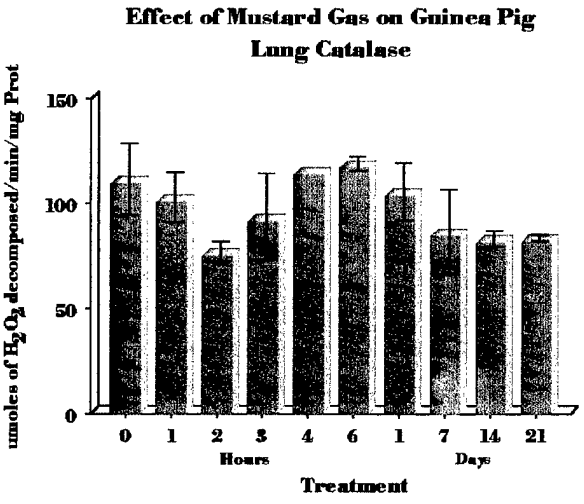

FIG. 5

Specific aim 3: To determine whether CEES exposure causes activation of sphingomyelinase and accumulation of ceramides in guinea pig lung.

The results this study clearly demonstrated that both the acidic and neutral sphingomylinase activities were increased 4-5 fold after CEES treatment. As the control level of acidic sphingomylinase activity was much higher than the control level of neutral sphingomylinase, we could see much higher activity of acid sphingomylinase compared to neutral sphingomylinase after CEES treatment (Figs. 6-7). Both acid and neutral sphingomyelinase activities started to increase along with the increase of TNFα but gives a maximum peak within 4 to 6 hr after CEES exposure. The level of sphingomyelinase decreased rapidly to come back close to normal level within 24 hours.

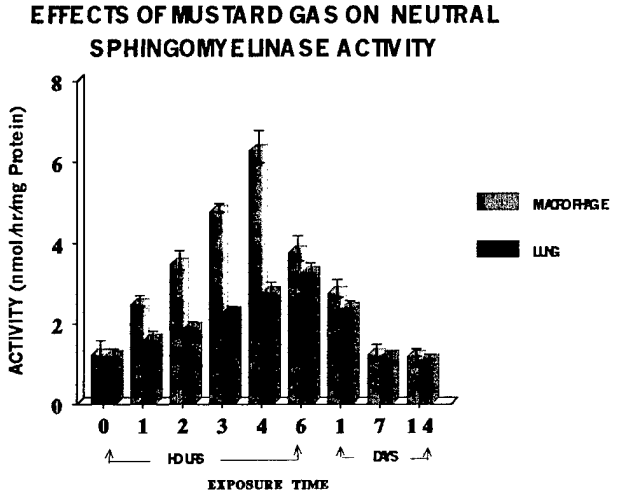

FIG. 6

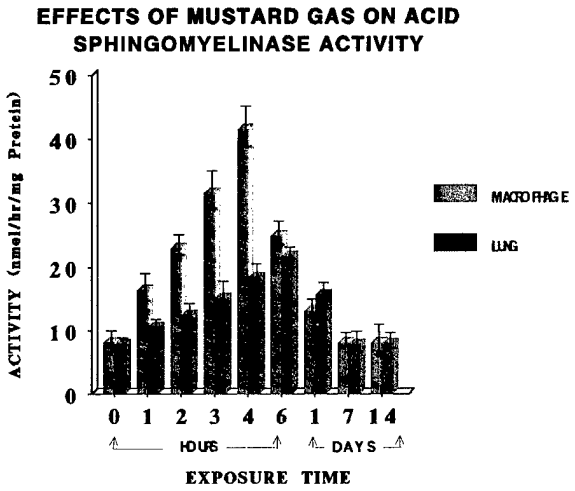

FIG. 7

The ceramides accumulation followed by CEES exposure demonstrated a biphasic pattern. Within 1 hour of CEES exposure, ceremides level became very high and gives a peak accumulation within 2 hours (Figs. 8-9). After 2 hours, there was some decrease in the ceramides level but again the level increased to a very high level and remained almost steady even upto 14 days (slight decrease in ceramides level at day 14).

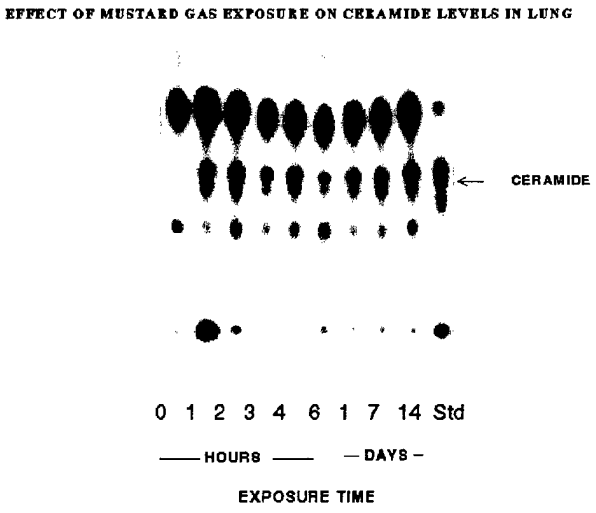

FIG. 8

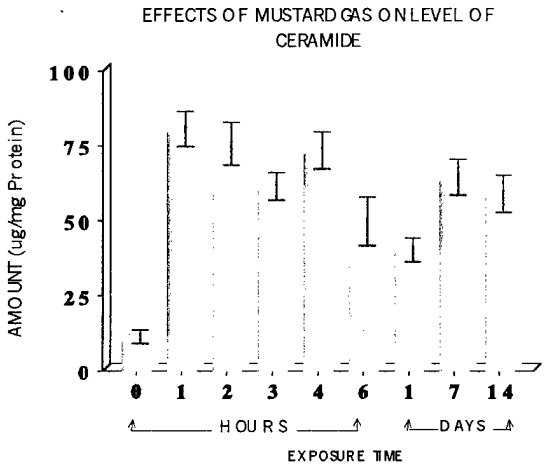

FIG. 9

Additional Studies Done Related to the Project but Not Included In Our Original Goals

1. Effects of Mustard Gas Exposure on NFkB Levels in Lungs

The NFkB activation was measured in the nuclear extracts of lungs after exposure to CEES. NFkB which is well known to oppose the TNFα mediated apoptosis, showed activation only upto 2 hours after CEES exposure (Fig. 10). Hence, we could see a biphasic effect of CEES on lung. After initial lung damage by TNF-α within 2 hours there was some recovery due to activation of NFkB. After 2 hours NFkB level went down, ceramide level increased and secondary lung damages were observed.

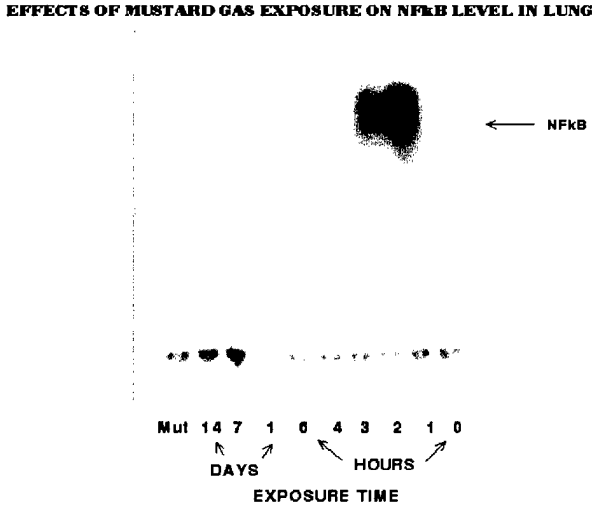

FIG. 10

2. Effects of N-acetylcysteine Treatment on the Mustard Gas Induced Lung damage (Induction of TNF-α and Ceramide Accumulation)

- The guinea pigs were given N-acetylcysteine in drinking water (0.5 gms per day) for three days before injection of mustard gas. After 1 hour of mustard gas injection, the guinea pigs were sacrificed. TNF-α and ceramide content of lung were assayed. The results indicated that the TNF-α level was decreased about 45-50% in the N-acetylcysteine treated animals compared to the experimental animals after mustard gas exposure.

- The effect of N-acetylcysteine on the mustard gas induced accumulation of ceramides was more pronounced. About 70% decrease in the accumulated ceramide levels after mustard gas injection were observed in the presence of N-acetylcysteine compared to the experimental animals.

3. Evidence of CEES-Induced Lung Injury by Histological and Ultrastructural Studies

Light microscopy studies showed evidence of severe lung damage, including edema, congestion, hemorrhage and inflammation after exposure to CEES.(Fig. 11A and 11B). The electron microscopic studies demonstrated damage of Type II cells and spillage of alveolar Type II cells in alveolar space (Fig. 11C and 11D).

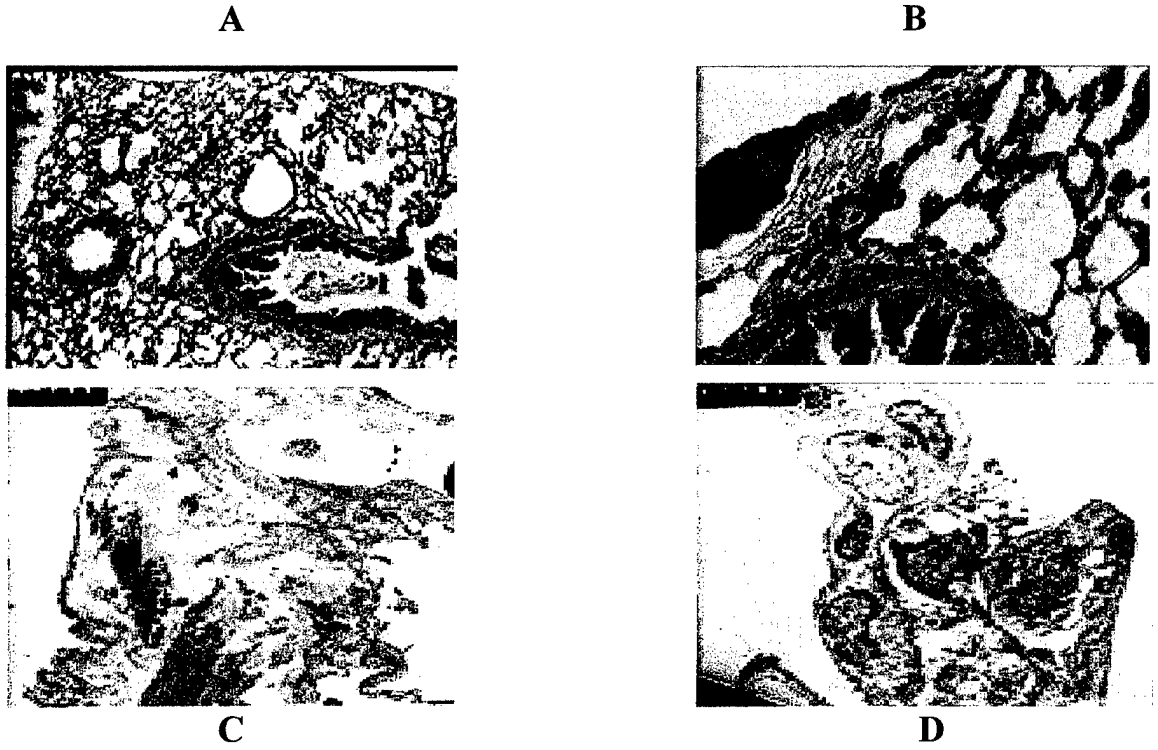

FIG. 11

4. Evidence of Hair loss after exposure to CEES and Beneficial Effects of NAC

After mustard gas exposure we observed the loosening of hair in the experimental animals (Fig. 12). The hairs came out very easily out of skin of CEES exposed animals (extreme left) compared to the hair in the control animals (middle). This CEES mediated hair loss could be significantly inhibited by treatment with N-acetylcysteine (extreme right). This hair loss can be used as an evidence of CEES toxicity. This needs thorough investigation before we could use hair loss as a Biomarker for CEES toxicity.

FIG. 12

5. Evidence of Obesity Like Syndrome by CEES Exposure:

After CEES exposure we have observed significantly much more body weight gain in the experimental animals compared to the control group (Fig. 13).

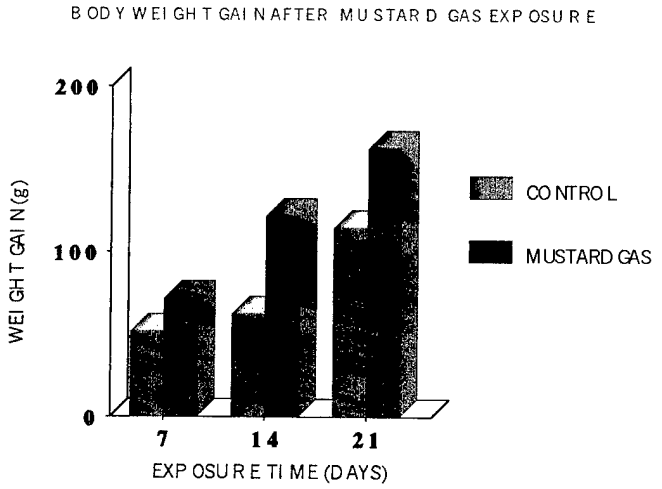

FIG. 13

In this case also, we need thorough investigation before using hair loss as a biomarker for CEES toxicity.

KEY RESEARCH ACCOMPLISHMENT:

- Both light and ultrastructural studies revealed evidence of severe of lung damage, including edema, congestion, hemorrhage, and inflammation at as early as 1 hr of CEES exposure. After 6 hours there were some recovery of the damage and in long term we could see diffused inflammation and apoptic nuclei in lung.
- After intra tracheal injection of CEES to guinea pigs, the TNFα level increased sharply within one hour of exposure.
- TNFα level started declining after one hour and came back to basal level within 24 hours.
- The stimulation of sphingomyelinase activity followed the accumulation of TNFα giving a peak within 4 to 6 hours after CEES exposure.
- Though both the acid and neutral sphingomyelinase activities were stimulated, the level of acid sphingomyelinase was found to be much higher after CEES exposure.
- As the sphingomyelinase activity increased, the accumulation of ceramides started. Ceramide level increased within one hour of CEES exposure and after a slight fall in the ceramide level at 3-6 hours, increased again and remained at high level even upto 14 days after CEES exposure.

- Slight drop in the ceramide level in between 3-6 hours might be due to the activation of NFkB, which showed a sharp transitional activation at 1 to 2 hour after CEES exposure. The activation of NFkB coincided with the increase of TNFα in lung tissue.

- The oxygen defense system of lung after CEES exposure were affected by CEES exposure.

REPORTABLE OUTCOMES:

1. **Abstract** : " Mustard Gas, Free Radical Status and Lung Injury", S. Mukherjee, D. Chatterjee and S.K. Das. Annual meeting of American Society for Biochemistry and Molecular Biology, Orlando, Florida. (will be held from March 31-April 4, 2001).

2. **Chapter in Book**: "The therapeutic use of antioxidant liposomes" Stone W., Mukherjee S., Smith M. and Das S.K., In "Liposomes – Methods and Protocols". Humana Press.

3. **Prospective Papers**:

   A. "Lung injury by mustard gas exposure; identification of signal transduction pathways using guinea pig model", D. Chatterjee, S. Mukherjee and S.K. Das.

   B. "Reversal of lung injury after mustard gas exposure by N-acetylcysteine", D. Chatterjee, S. Mukherjee and S.K. Das.

   C. "Biomarkers for mustard gas exposure", D. Chatterjee, S. Mukherjee and S.K. Das.

   D. "Modulation of oxygen defense system in lung after mustard gas exposure", S. Mukherjee, D. Chatterjee and S.K. Das.

4. **Development of the animal model**:

   We have developed guinea pig model for studying mustard gas exposure. As guinea pigs are closer to human system than other primates, and it is difficult to get human volunteers for mustard gas study, guinea pig model will be ideal for our study to develop antidotes for mustard gas.

CONCLUSIONS:

The results presented here clearly demonstrated the involvement of signal transduction pathway in the mustard gas mediated lung injury. We need additional work to confirm the findings reported here. We need to initiate our studies on specific aim 4 and 5 as outlined in the original proposal concerning lung surfactant, β- adrenergic receptors and development of antidotes (anti oxident liposomes and others) for the mustard gas mediated ARDS. There will be no need of change of our original proposal. Only we might include three additional parameters in our extended study. The first one is a direct demonstration of the lung damage by also studying the leakage of ^{125}I-BSA from lung after CEES treatment. Other two parameters are

the effects on hair loss and body weight gain of the animals. Those two parameters will be biomarkers for the mustard gas effects as it will not be possible to test the lung condition or other parameters from lung in human subjects during the human trial of those antidotes.

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Section C

William Stone

Our long-term goals are to prepare and characterize antioxidant liposomes, characterize the toxicity of HMG to macrophages and to evaluate the potential therapeutic role of antioxidants. We have found that stimulated macrophages are more susceptible to HMG than un-stimulated macrophages. A very low level of LPS (20 ng/ml) greatly enhances the toxicity of HMG at concentrations greater than 400 micromolar. The cytotoxic interaction of LPS and HMG reaches a maximum 12 hours after exposure. Low levels of HMG (100 micromolar) were found to partially inhibit the secretion of nitric oxide (NO) in stimulated macrophages and levels higher than 300 micromolar were found to completely inhibit NO production. HMG did not, however, directly inhibit inducible nitric oxide synthase (iNOS) activity but did block the cellular synthesis of the iNOS enzyme as measured by Western blots. Oxidative stress in macrophages was also measured by use of a fluorescent microplate reader. Macrophages treated with HMG were found to have an increased level of oxidative stress. N-Acetyl cysteine (a potent antioxidant) was able to protect stimulated macrophages from HMG toxicity. Our results suggest that cytokines that stimulate macrophage activation may enhance the toxicity of mustard gas. Moreover, mustard gas may be a potent *in vivo* inhibitor of nitric oxide production. N-Acetyl-cysteine may be an important antioxidant that affords protection against mustard gas toxicity. Future work will examine the effects of antioxidant liposomes containing N-acetyl cysteine as well as lipid soluble antioxidants such as tocopherols.

(no proprietary or unpublished material)

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Introduction

Considerable evidence suggests that mustard gas toxicity is associated with an increased generation of damaging free radical production [1-4]. Antioxidant liposomes may provide a unique therapeutic strategy for mustard gas exposure because: (a) the antioxidants are nontoxic and could, therefore, be used at the earliest stages of toxicity (b) the liposomes themselves are composed of nontoxic, biodegradable and reusable phospholipids; liposomes are preferentially taken up by the reticuloendothelial system which is an early target of mustard gas toxicity; (d) chemical antioxidants are relatively inexpensive and a wide range of nontoxic commercial antioxidants are available. The long term goals of this two year project are to further define the mode of action of mustard gas and to develop a therapy using liposomes containing both lipid and water soluble antioxidants. These goals are consistent with the USAMRMC Medical Chemical Defense Research Program interests in the area of defense against chemical agents.

Macrophages are a particularly useful cell type in which to study the links between mustard gas toxicity, antioxidants and inflammation. Macrophages phagocytize liposomes that can, therefore, be used to deliver drugs or nutraceuticals to this cell type. The antioxidant content of macrophages can be easily manipulated by incubating these cells with liposomes containing lipid and water-soluble antioxidants.

Our specific aims are to:

- Prepare and characterize antioxidant liposomes
- Study the influence of antioxidant liposomes on half-mustard gas (HMG or 2-chloroethyl ethyl sulfide) toxicity to macrophages
- Evaluate the influence of antioxidants on HMG toxicity to macrophages

Body

Preparation of Antioxidant Liposomes

We have prepared antioxidant liposomes with a lipid soluble antioxidant in the form of either alpha-tocopherol or gamma-tocopherol (different forms of Vitamin E). Two techniques have been used to accomplish this goal. Small unilamellar liposomes were made by sonication. These liposomes have a diameter between 200 to 250 A°. These liposomes are not, however, useful for efficient capsulation of water-soluble solutes. Large unilamellar liposomes were also prepared by extrusion through polycarbonate membrane. We have found that liposomes prepared by extrusion are much less susceptible to *in vitro* oxidation during the preparation procedures than liposomes prepared by sonication. Experiments have been initiated to measure the antioxidant capacity of the tocopherol containing liposomes. We are currently developing novel

methods for characterizing the antioxidant capacity of liposomes since currently available methodologies have been found to be unsatisfactory.

LPS Stimulated RAW264.7 Macrophages Are More Susceptible to HMG Toxicity

As shown in figure 1,we have found that lipopolysaccharide (LPS) stimulated RAW264.7 macrophages (24 hour) are more susceptible to HMG toxicity (24 hr) than resting macrophages. In figure 1, cell viability was measured using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This assay is widely used assay to measure cell viability is based on reduction of the tetrazolium salt (MTT) by actively growing cells to produce a blue formazan product with absorbance at 575 nm. [5, 6]. LPS from Gram-negative bacteria is known to trigger a variety of inflammatory reactions such as the release of pro-inflammatory cytokines. LPS binds to CD14 and initiates a complex signal transduction pathway also involving the Toll receptor family. In addition, LPS activates NFkappaB, which activates the transcription of NO synthase (see below).

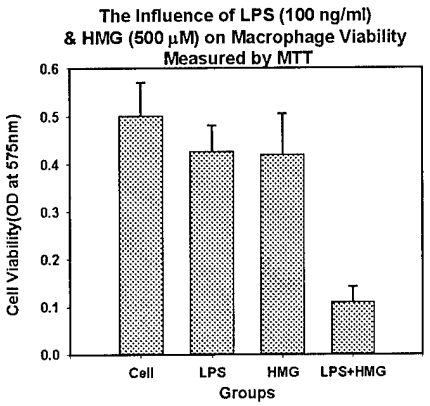

Figure 1

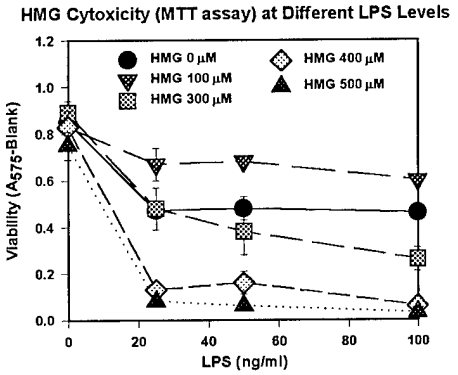

Figure 2

Characteristics of HMG Toxicity to LPS Stimulated RAW264.7 Macrophages

The data in figure 2 show that very low levels (20 ng/ml) of LPS (24 hr) dramatically enhance the toxicity of HMG (24 hr) to macrophages at concentrations > 400 μm. Figure 3 indicates that the cytotoxic interaction between LPS and HMG is at a maximum 12 hours after exposure.

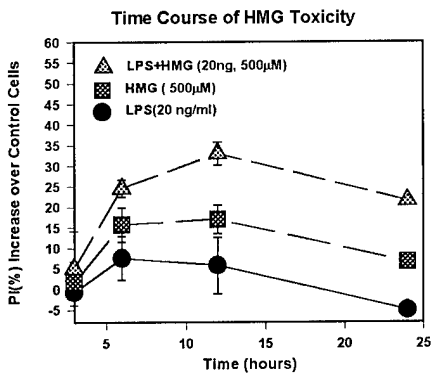

Figure 3

HMG Inhibits NO Production in Stimulated Macrophages

As mentioned above, LPS induces the synthesis of iNOS in RAW264.7 macrophages. The data is figure 4 indicates that NO production (as measured by nitrite secretion into the cell medium) is induced by 24 hr exposure to LPS concentrations as low as 20 ng/ml. It is also evident that HMG exposure (24 hours) to the macrophages dramatically decreases the secretion of NO (figure 4).
Low levels of HMG (100 μM) partially inhibit NO production whereas levels higher than 300 μM completely inhibit NO production. Additional data have shown that HMG does not directly inhibit iNOS activity and that HMG inhibits iNOS transcription/translation (Western blots).

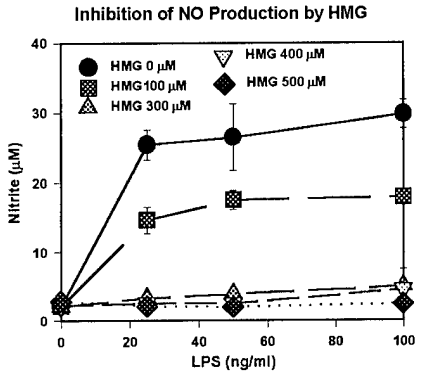

Figure 4

Oxidative Stress by HMG

We have developed a very useful method to measure free radical generation in cultured macrophages using a fluorescent microplate reader. This technique is based on the observation that free radicals promote the oxidation of non-fluorescent dichlorofluorescin (DCFH) into fluorescent dichlorofluoroscein (DCF). In stimulated macrophages, DCFH oxidation is primarily due to the generation of NO from iNOS (Imrich et al., Nitric Oxide:Biology and Chemistry, 1997). Figure 5 indicates that LPS treated macrophages show a dramatic induction in the rate of DCFH oxidation. The dose response data in figure 5 indicate this response is saturated at about 20 ng/ml of LPS. The oxidation of DCFH is most likely due to the LPS induction of NO production

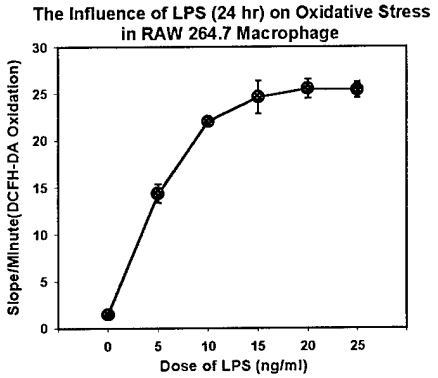

Figure 5

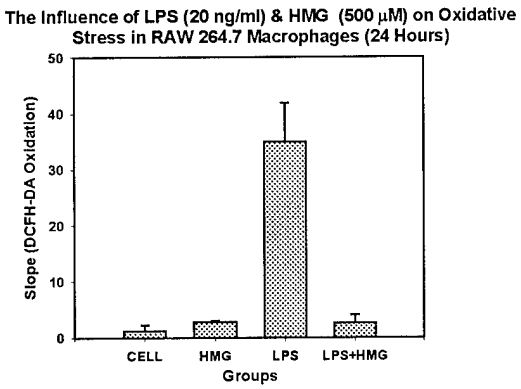

Figure 6

(see figure 4). In support of this notion, figure 6 shows that HMG is able to inhibit the rate of DCFH oxidation. It is significant that macrophages treated only with HMG show a significant induction in the production of free radicals.

As shown in figure 7, LPS stimulated macrophages treated with ebselen, a specific inhibitor of iNOS, show a marked inhibition in the rate of DCFH oxidation. These data further support the view that NO production is responsible for most DCFH oxidation in stimulated macrophages.

Effects of N-Acetyl Cysteine (NAC) on HMG Toxicity and NO Production

NAC is a readily available water-soluble antioxidant that is known to be safe for human use since it is currently used (in high doses) to counteract acetaminophen toxicity. Three experiments were performed in which: (1) NAC was added to macrophages with LPS and HMG (no pretreatment time); (2) NAC added 5 hours after treating macrophages with LPS and HMG (5 hour post-treatment); (3) NAC was added 5 hours before treatment with LPS and HMG. Figure 8 shows that, when10 mM NAC is added with LPS and HMG, there is a dramatic decrease in the toxicity of HMG with LPS as measured by the MTT assay. When administered 5 hour before HMG and LPS treatment NAC also affords significant protection. However, when administered 5 hours after HMG, NAC is less effective. Additional data indicates that NAC does not protect iNOS activity in HMG and LPS treated macrophages.

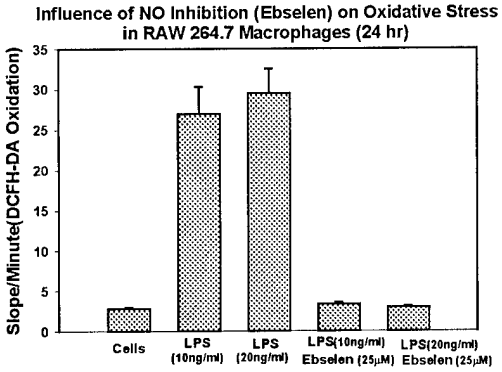

Figure 7

Key Research Accomplishments

- Cytokines that stimulate macrophage activation may enhance the toxicity of mustard gas.
- Mustard gas may dramatically inhibit in vivo iNOS activity.
- Mast cell activation is promoted by iNOS inhibitors and activated mast cells release cytokines that activate macrophages.

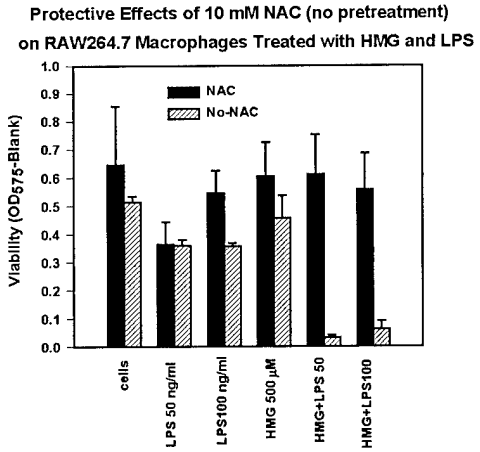

Figure 8

- NAC may protect in animal models and in human exposure to mustard gas. NAC is inexpensive, nontoxic and can be taken orally.
- Antioxidant liposomes with NAC and other antioxidants may be even more effective

Reportable Outcomes

- Stimulated macrophages are more sensitive to HMG toxicity
- HMG treatment to un-stimulated macrophages is associated with increased oxidative stress
- HMG is a very effective inhibitor of iNOS enzyme production but does not inhibit iNOS activity directly
- NAC protects against HMG toxicity in LPS stimulated macrophages
- NAC does not protect iNOS inhibition caused by HMG treatment

Conclusions

NAC has been found to be very effective in preventing HMG toxicity to stimulated macrophages. This is an important observation with major implications for the development of strategies to deal with terrorist use of mustards gas on either military or civilian populations. NAC is readily available in most pharmacies and, therefore, the logistics of obtaining it for emergency use at any location is straightforward. Specialized storage conditions are not required. In future experiments we hope to further define the molecular mechanism for the HMG mediated inhibition of NO production. This is an important issue since NO is an important biomolecule with numerous physiological roles.

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Appendices

Section D

Keith Crawford

Myeloid cells are a unique group of immune cells, which are ubiquitously located throughout the body. These immune cells have principal roles in innate and adaptive immunity, and are highly secretory cells capable fo releasing a variety of molecules ranging from complement proteins to oxygen metabolites. Uncontrolled releases of these secretory products cause extensive tissue damage. Complement has long been known to be activated at sites of tissue injury and it is widely believed that the uncontrolled activation usually has deleterious effects. Complement damages tissues directly by deposotion of the membrane attack complex and by providing cell-bound ligands, including C3 and C4 binding of PBMC bearing complement receptors. Complement can also amplify injury by the production of anaphylatoxins C5a and C3a, leading to the activation and influx of inflammatory cells. The objective of this research is to determine the role that two myeloid cells, macrophages and dendritic cells, play in tissue damage due to mustard gas exposure and demonstrate that a novel liposome-antioxidant formulation decreases this damage. Our initial focus is to determine baseline kinetics of complement production in dendritic cells/monocytes. We will subsequently investigate the effect of half mustard exposure on sysntheis and secretion of the complement (C2, C3, C4, C5, and factor B).

Table of Contents

Introduction:

The complement systems comprise about 30 interacting components and regulatory proteins, mostly in the plasma and extracellular fluid, but also on cell surfaces (1). Activation of the complement system is through three arms: (a) the classical pathway, activated primarily by aggregated antibody (usually in antigen-antibody complexes) and consisting of C1, C4, and C2, surfaces, including those of gram-negative bacteria and yeast; (b) aternative and (c) the lectin pathway, involving mannose-binding protein, and at least two interacting proteins, MASP-1 AND MASP-2. These three pathways, when activated, cleave and activate C3, which, in turn, results in cleavage and activation of C5. There are inhibitors and regulators of complement activation, both in the fluid phase and on cell surfaces, sot that there is tight control of the activation process. The major fragment of activated C5, C5b, induces the assembly of the membrane attack complex (MAC) containing C5b, C6, C7, C8, and poly C9. The MAC acts in a manner similar to lymphocyte surface perforin to produce holes in the target cell membranes, cause lysis.

Body:

A large number of biologic functions involve in host defense against pathogenic microorganisms and their metabolic products are mediated by complement proteins and their proteolytic cleavage products. It is now clear that the complement systems participates in opsonization of and clearance of microorganism (1, 2)), in the generation of biologically active peptides that mediate smooth muscle contraction and increased vascular permeability, leukocyte chemotaxis and aggregation and mobilization. The major relevance of the complement system to human health was first pointed out by the increased susceptibility to bacterial infections (3) and autoimmune disease (4) of rare persons with inherited deficiencies and, more recently, of mice engineered inherited deficiencies of complement proteins. Humans deficient in C1q or C4 or, to a lesser extent, C2 have an increase incidence of autoimmune diseases, particularly systemic lupus erythematous (1), knockout out also have lupus-like disease (5) further suggesting that the complement systems contributes to protection against autoimmunity. Thus, the complement system participates in and is essential for optimal functioning of both the adaptive and innate arm of the immune system.

Although the liver is the primary site of synthesis of circulating complement component (6), a number of other tissue also sythesize and release complement protein (7). In particular, monocytes/macrophages have been shown to produce C2 (8), C3 (9), C4 (10), C5 (11) and factor B (12). It is probable that they do not synthesize C6, C7, C8, or C9 (13), although this is controversial (14). Mo also synthesize the regulatory proteins C1 inhibitor (15) and

factor H (16). The production of complement proteins at local sites clearly is critical for the tight regulation of inflammatory and immune responses. The controlled activation and release of complement has no damaging effect on lung, skin, or other tissues of the body, while uncontrolled activation, the inability to clear complement, and decreased concentrations of complement inhibiters are most likely the principal factors causing local cytopathogenesis. Previous studies (unpublished) suggest that treatment of pan-activated (i.e. PMA, LPS, IFNγ) myeloid cells with N-acetylcysteine (NAC), prior to exposure or shortly following exposure, decreases the release of secretory products.

The mechanism by which MG activates myeloid cells and induces the release of secretory products is poorly understood, but it is highly probable that the activation of myeloid cells is similar to that of other pan-activators. Thus, treatment with NAC should decrease mustard induced-cytopathogenesis by suppressing the release of complement or inhibiting complement activation by deactivating serine proteases. These studies shall provide the framework, by which, an antidote for MG exposure is developed; in addition to providing a better understanding of the mechanisms responsible tissue damage after acute exposure or the autoimmune-like symptoms that occur in subjects years after exposure.

Key Research Accomplishments:

Because of limited availability of assays for the study of the complement system, we have developed assays to measure principal complement proteins, complement regulatory proteins, and other secretory products synthesized by the myeloid cells. These assays are the Dot Blot, the Western Blot, the ELISA, and Single-cell (SC)RT-PCR. The table below summarizes the secretory products, which will be assessed and their stage readiness.

Secretory Products	Dot Blot	ELISA	Western Blot	SC RT-PCR
C1				
C1 inhibitor	Complete	Completed	Completed	
C4	Completed			
C2	Completed			
C3	Completed	Completed	Completed	
Factor B	Completed			
Factor H	Completed	Completed	Completed	
Factor I	Completed	Completed	Completed	
C5	Completed	Completed	Completed	
C6	Complete			
C7	Completed			
C8	Completed			
C9	Completed			
Elastase				
Caspase 3				

Reportable Outcomes:

C3 synthesis by DC and Mo (Appendix I). We examined C3 production by separated and unseparated DC, Mo, cultured in serum-free medium for 24 hours. ELISA was used to measure C3 levels in cultured supernatants. As seen in figure 1, C3 production by DC was significantly higher in the separated DC population than when together with Mo or Mo alone. This is of great significance since DC are found in large numbers in the skin and lung. However, once activated, DC detach and migrate, in an activated stated, to regional lymph tissue.

C1INH synthesis by DC and Mo (Appendix II). C1 inhibitor is a major regulator of the Classical Complement Pathway. The synthesis of C1INH by combined and separated DC and Mo was examined. There was minimal synthesis in inactivated states after 72 hours. However, IFNγ had a dramatic effect, increasing C1INH synthesis by DC greater than that of the Mo. Surprisingly, when the cells are combined, the level of C1INH is 2 to 3 folder higher than DC or Mo, respectively.

Conclusions:

It is possible that when both populations are together, adequate levels of C1INH are released allowing for tight regulation of the classical pathway. However, when C1INH production is exhausted or the levels of production are decreased because of massive migration of DC from the site of exposure, the Classical Pathway cascade begins. Collectively, the loss of Classical Pathway control and the increase in C3 levels allow for the uncontrolled activation of the complement cascade and subsequent tissue damage.

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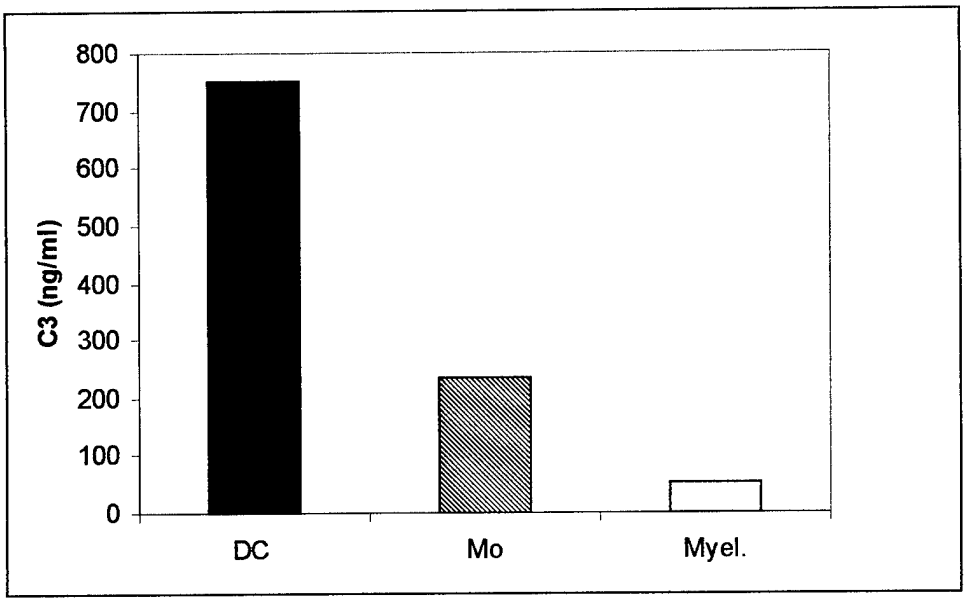
Appendix

I



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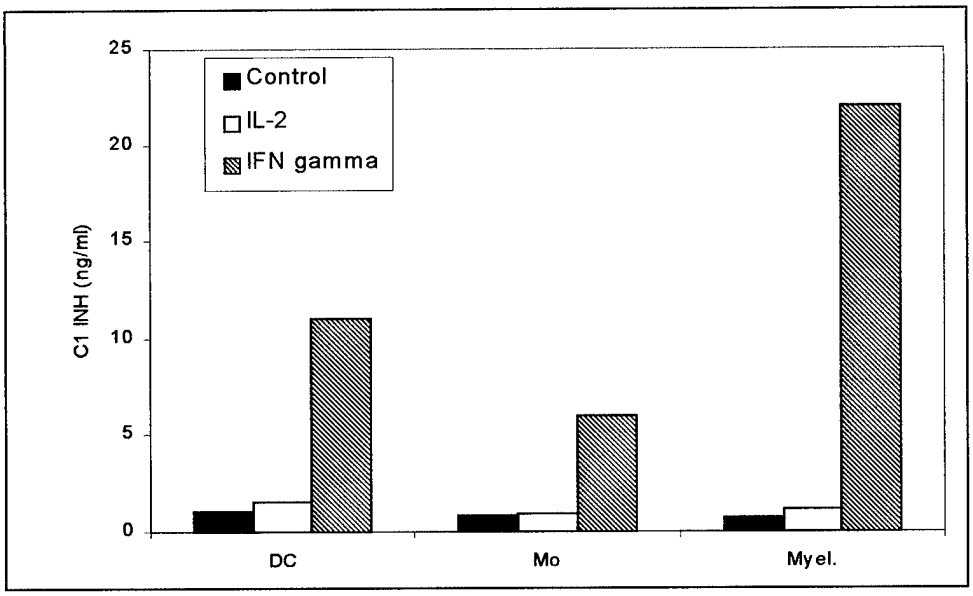

The First Mustard Consortium-Crystal City Virginia October 20th, 2000

- Summation of Scientific Work by the Consortium
- Article: W. Stone, S. Mukherjee, S.K. Das, and M. Smith: The Therapeutic Use of Antioxidant Liposomes

Scientific Investigators in the Mustard Consortium:
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Keith Crawford, M.D., Ph.D.
Peter Ward, M.D.
William Stone, Ph.D.
Salil Das, DSc
Gerd Till, M.D.
Carl Alving, M.D.
Janos Szebeni, M.D. Ph.D.

Section A: A Summary of the research results for the Consortium

The Role of Myeloid Cells in Mustard Gas induced Cytopathogenesis.

Keth Crawford, M.D., Ph.D.
Center for Blood Research, Harvard Medical School

It has been known for decades that mustard gas and its derivatives wreck havoc on the immune systems, but how this is done is poorly understood. Myeloid cells directly interact with B, T, NK, and dendritic cells to initiate specific immune responses or they can independently initiate immunity. These cells survey various tissues of the body and identify alterations of plasma membranes. These alterations signal myeloid cells to engulf damaged cells and complete the degradation process. Since myeloid cells are intimately involved in the activation of innate and adaptive immune responses, we will determine how mustard derivatives effect myeloid cell function

MECHANISMS AND PREVENTION OF LUNG INJURY CAUSED BY MUSTARD GAS

Gerd O. Till, M.D. and Peter A. Ward, M.D.
Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109.

There is accumulating evidence that exposure to sulfur mustard gas results in a variety of tissue-damaging outcomes, especially in skin and lung, although our understanding of the mechanisms responsible for this injury is quite limited. We now have shown that a version of mustard gas, 2-chloroethyl ethyl sulfide ($Cl\text{-}CH_2CH_2SCH_2CH_3$, "half mustard gas," HMG), when instilled into the airways of rats, induces acute lung injury characterized by extensive intrapulmonary hemorrhage and edema. These effects were dependent on the dose of HMG and proportional to the time after exposure to HMG. For example, at 4 hours after the intrapulmonary deposition of 6 µl HMG per kg body weight, lung injury values (as determined by the extravasation of radiolabeled bovine serum albumin) increased seven-fold above background values. Interventional studies showed that the development of HMG-induced lung injury was dependent on the availability of both neutrophils and complement. Since the appearance of lipid peroxidation products in plasma of HMG-treated animals suggested involvement of toxic oxygen metabolites, N-acetyl-L-cysteine (NAC), a well-known anti-oxidant, was administered intravenously and was shown to have powerful, protective effects related to the dose administered and the time of its administration. NAC administration could be withheld for one hour after exposure of lung to HMG and still

confer high degrees of protection. These data confirm that the rat lung is highly susceptible to HMG-induced acute lung injury and that injury is substantially reduced when the anti-oxidant, NAC, is given intravenously.

The Effects of Mustard Compounds on The Guinea Pig Lung

Principal Investigator: **Salil K. Das, Sc.D., D.Sc.**

Research Associates: **Diptendu Chatterjee, Ph.D.**

Shaymali Mukherjee , Ph.D.

1. Individuals exposed to mustard gas develop lung injury. In order to develop some means to protect them from such injury, we need to know how mustard gas damages lung tissue. Since human volunteers cannot be used for such studies, , we need to develop a good animal model. Our laboratory has established that the guinea pig's lungs are similar to human lungs and can be used to study lung structure and function.

   We have used the guinea pig model to study the effects of mustard gas on lung structure and function. Preliminary results indicate that very short-term exposure (one to two hours) causes lung damage (inflammation, hemorrhage, edema and alveolar congestion etc.) accompanied by biochemical changes which kill lung cells. The major biochemical change is the accumulation of a special kind of lipids called ceramides which are known to be responsible for programmed cell death in inflammatory diseases. Our immediate goal is to learn in detail the pathway by which ceramides accumulate and the consequences they exert on lung cells. . Our ultimate goal is to develop an antidote to stop the accumulation of this lipid and thus prevent the adverse effects of mustard gas on lung.

2. Besides initiating lung injury, mustard gas causes several other health problems. There are no known biomarkers available for the diagnosis of mustard gas toxicity in humans. Our goal is to develop a biomarker. Our preliminary observations suggests that guinea pigs exposed to mustard gas develop:

   a. Loosening of hair folicles
   b. Obesity like syndrome
   c. Heart injury

   It is important that we continue our efforts in this area to confirm these observations with an ultimate goal to develop a biomarker for mustard gas related toxicity.

Interaction of antioxidant liposomes with the complement system

Janos Szebeni, Janos Milosevits, Lajos Baranyi and Carl

The potential therapeutic benefit of a-tocopherol (aT) and glutathione-containing antioxidant liposomes (AL) against diseases involving oxidative tissue damage, such as mustard gas-induced lung injury and ARDS, may depend, among others, on the influence of AL on the complement (C) system. In particular, C activation with consequent opsonization of vesicles are expected to decrease the circulation time of parenteral AL, as well as to counteract the therapeutic efficacy of antioxidants by enhancing the inflammation process via production of C3a and C5a anaphylatoxins. On the other hand, AL capable to inhibit C activation may have the double benefit of extended circulation time and inhibition of the propagation of oxidative damage-related inflammation.

The initial studies reported here focused on large multilamellar DMPC/DMPG/aT/Cholesterol-containing AL, based on our previous observations that these liposomes mimic small unilamellar polyethylene-glycol (PEG)-coated, "stealth" liposomes (Doxil) in terms of capability to activate C and to cause C activation-related acute physiological effects in pigs and rats (Szebeni et al., J. Liposome Res., In press). We carried out in vitro incubations of AL with normal human sera and measured C consumption (CH50) and the production of the C terminal complex (SCSb-9) as indices of C activation, and also analyzed AL-induced respiratory burst in human neutrophil leukocytes and monocytes by flow cytometry, in the presence and absence of serum. The data indicated highly variable C activation by various formulations of AL with or without (up to) 50% membrane aT, suggesting that AL may act as a proinflammatory agent in certain individuals unless C activation is not controlled. Consistent with these observations, in the presence of serum, AL also caused intense early (minutes), as well as late (1-2 h) changes in intracellular redox potential of granulocytes and monocytes, attesting to the biological relevance of C activation. These changes were modulated in a complex fashion by altering the aT content of liposomes. In conclusion, our initial data raise the possibility that C activation by AL could counteract its therapeutic benefit via anaphylatoxin

production and subsequent activation of granulocytes. Thus, elaboration of a truly C-stealth AL liposome formulation seems not only desirable for achieving the mentioned theoretical benefits, but appears actually essential to prevent possible aggravation of inflammation. Our data may also explain earlier observations on a proinflammatory effect of intratracheally administered "stealth" AL.

Therapeutic Uses of Antioxidant Liposomes

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1. Introduction

This review focuses on the use of antioxidant liposomes in the general area of free radical biology and medicine. The term "antioxidant liposome" is relatively new and refers to liposomes containing lipid soluble chemical antioxidants, water-soluble chemical antioxidants, enzymatic antioxidants, or combinations of these various antioxidants. The role of antioxidants in health and disease has been extensively discussed, and many excellent reviews and books are available (1-3). Antioxidant liposomes hold great promise in the treatment of many diseases in which oxidative stress plays a prominent role. Oxidative stress is a physiological condition in which the production of damaging free radicals exceeds the *in vivo* capacity of antioxidant protection mechanisms to prevent pathophysiology. Free radicals are molecules with unpaired electrons, often highly reactive and damaging to biological systems. The biological membranes of subcellular organelles are a major site of free radical damage but proteins and DNA are also significant targets. Moreover, free radicals can alter cellular signal transduction pathways and stimulate the synthesis of inflammatory cytokines. Oxygen radicals and other reactive oxygen species (ROS) arise from the single electron reductions of oxygen.

(1) $O_2 + e^- \rightarrow O_2^{*-}$

(2) $O_2^{*-} + e^- + 2H^+ \rightarrow H_2O_2$

(3) $H_2O_2 + e^- + H^+ \rightarrow H_2O + OH^*$

(4) $OH^* + e^- + H^+ \rightarrow H_2O$

(5) $O_2^{*-} + NO \rightarrow ONOO^-$

In addition, the superoxide radical (O2*-) can react rapidly with nitric oxide to yield peroxynitrite as shown in reaction (5). Peroxynitrite is a reactive nitrogen oxide species (RNOS) that can also cause damage to DNA, proteins, and membranes. Moreover, ONOO- is likely to be generated during inflammation and the killing of bacteria.. Free radicals are generated in both the aqueous and lipid compartments of cells and, to minimize their damaging effects requires both lipid and water soluble antioxidants. Nevertheless, the potential clinical use of such a bifunctional liposomes has been extremely limited (4).

A primary use of antioxidant liposomes has been to define the molecular mechanism of action for various antioxidants (5-13). Antioxidants such as butylated hydroxytoluene (BHT) and alpha-tocopherol have also been used to prevent the oxidation of unsaturated fatty acid moieties in the phospholipids of liposomes during storage (14) or sonication (15). This chapter, however, focuses on the potential therapeutic uses of antioxidant liposomes. This is a rapidly evolving area of medical research not extensively reviewed. Most of the research to date has been accomplished using *in vitro* cell culture systems or animal models. Very few clinical trials have been attempted yet obvious medical situations exist (e.g., influenza infection as discussed below) in which antioxidant liposomes have enormous health-related significance. The preparation of antioxidant liposomes that can be targeted to specific sites in the body is also a promising area but awaits further research. Most chemical antioxidants are phytoceuticals whose

properties have already been extensively studied and are generally regarded as nontoxic and safe for human consumption (1). In the following sections, we will first review the varieties of antioxidants that have either been used in antioxidant liposomes or hold the promise of such utilization. We will then focus on issues relating to the modes of administration and lastly describe the clinical uses of antioxidant liposomes for diseases in which oxidative stress plays a major role. Major emphasis will be placed on the use of antioxidant liposomes for pulmonary diseases.

2. Lipid Soluble Antioxidants

The lipid soluble antioxidants that can be incorporated into liposomes include vitamin E (tocopherols and tocotrienols) (16), ubiquinones (17), retinoids (18-20), carotenoids (21,22), lipid soluble flavonoids (e.g., quercetin, hesperetin, naringenin) (23), tamoxifen (24,25), as well as synthetic lipid soluble antioxidants such as BHT, tertiary-butyl-hydroquinone (TBHQ), and probucol. Tocopherols can readily be incorporated into both monolayers of unilamellar liposomes in a monomeric form (16). Furthermore, tocopherol in liposomes can undergo spontaneous intermembrane transfer to an acceptor membrane without the fusion of the tocopherol liposome (16). This intermembrane transfer is more pronounced when the tocopherol liposome contains polyunsaturated fatty acids (16). RRR-alpha-tocopherol and RRR-alpha-tocotrienol are forms of vitamin E that have the same aromatic chromanol head group but differ in the structure of their hydrocarbon tails. RRR-alpha-tocotrienol is, however, a better peroxyl radical scavenger than RRR-alpha-tocopherol in phosphatidylcholine liposomes (26).

Beta-carotene (a carotinoid) can be incorporated into liposomes to a maximum of about

0.5 mol% (based on phospholipid) whereas tocopherol can be incorporated at levels as high as 30 mol%. The ability of beta-carotene in liposomes to inhibit free radical mediated lipid peroxidation appears, however, to be much lower than that of alpha-tocopherol (27). Beta-carotene at 0.45 mol% (of phospholipid) is, however, a more powerful inhibitor of singlet oxygen mediated lipid peroxidation than alpha-tocopherol at 0.45 mol% (28). Alpha-tocopherol at 4.5 mol% is, however, also effective at inhibiting both free radical lipid peroxidation as well as singlet oxygen mediated lipid peroxidation (28). Singlet oxygen can be generated by photosensitizes and this reactive oxygen species may contribute to light induced skin toxicity as well as the aging of skin.

The lipids used in the preparation of antioxidant liposomes also provide an opportunity to introduce antioxidant capacity into liposomes. For example, plasmalogens (1-alkenyl, 2-acyl-) phospholipids are thought to have antioxidant properties (29,30). Liposomes constructed with ethanolamine plasmalogen inhibit both iron and copper-dependent peroxidation in the presence of preformed lipid hydroperoxides (31). Koga et al. have synthesized a novel phospholipid containing a chromanol structure as its polar head group (12,32). This phosphatidyl derivative of vitamin E is almost as effective an antioxidant as alpha-tocopherol in unilamellar liposomes subjected to free radicals generated in the lipid phase. The potential therapeutic value of liposomes with antioxidant phospholipids has not been explored but this is an obvious area for future research.

A major advantage of antioxidant liposomes is their ability to simultaneously contain (and deliver) both water and lipid soluble antioxidants. This is particularly important in

the case of liposomes with both tocopherol (TOH) and ascorbate (Asc) since it has been demonstrated that ascorbate can regenerate tocopherol from the tocopheroxyl radical (TO*) (33).

(6) $TO^* + Asc \rightarrow TOH + Asc^*$

3. Water Soluble Antioxidants

The water soluble antioxidants that can be used in antioxidant liposomes include ascorbate (vitamin C), urate, glutathione, N-acetylcysteine (NAC), lipoic acid (or dihydrolipoic acid which is its reduced form), pro-cysteine, and water soluble flavonoids (as in pycnogenol). Dihydrolipoic acid is somewhat unique since it can quench peroxyl radicals generated both in the aqueous phase and in membranes (34). Chemical antioxidants generally act by donating an electron to a free radical (thereby quenching the free radical) or by serving as a substrate for an antioxidant enzyme. Glutathione, for example, is itself an antioxidant (6) and can also function as a substrate for glutathione peroxidase, a key (selenium containing) antioxidant enzyme that converts lipid hydroperoxides (LOOH) or H_2O_2 into the corresponding lipid alcohols (LOH) or H_2O. Chemical antioxidants can also be chelators of transition metal ions that catalyze lipid peroxidation reactions. Urate, which is present at very high concentrations in human plasma, is an excellent antioxidant that can both chelate transition metal ions and also quench aqueous free radicals (35).

4. Entrapped Antioxidant Enzymes

The application of antioxidant liposomes to problems of medical interest has primarily been with liposomes containing entrapped antioxidant enzymes. Recombinant biotechnology has provided the means to obtain large (i.e., commercial) quantities of human antioxidant enzyme but

these enzymes do not normally penetrate the plasma membrane of cells and have a short half-life when introduced into the body by intravenous injection. Turrens has reviewed the potential of antioxidant enzymes as *in vivo* pharmacological agents (36). The attachment of polyethylene glycol (PEG) to antioxidant enzymes increases their *in vivo* half lives and their effectiveness in preventing pulmonary oxygen toxicity in rats (37). The various procedures for preparing liposomes with entrapped antioxidant enzymes have been evaluated by Aoki et al. (38). This group and others (39) have found that positively charged liposomes have a superior trapping efficiency for superoxide dismutase (which has a negative charge).

Early work by Freeman et al. (40) has shown that porcine aortic endothelial cells treated with liposomes with entrapped superoxide dismutase (SOD-liposomes) can dramatically increase their cellular SOD levels and thereby protect the cells from oxygen induced injury. In a key paper, Beckman et al. (41) found that endothelial cells treated with liposomes containing entrapped superoxide dismutase and catalase (SOD+CAT-liposomes) can increase the cellular specific activity of these enzymes by at least 40-fold within 2 hours. These results are particularly important because endothelial cells are a major site for oxidative damage. Moreover, intravenous antioxidant liposomes would certainly make contact with vascular endothelial cells under *in vivo* conditions.

5. Modes of Administration

Antioxidant liposomes can be administered topically, intratracheally, intravenously, by inhalation in an aerosol form, or by intra-muscular injection. Topical administration can certainly be long term and is of considerable interest to the cosmetic industry in treating specific

skin disorders such as psoriasis. Alpha-tocopheryl acetate in liposomes has been found to have a better dermal absorption than free alpha-tocopheryl acetate (42). Topical administration of antioxidant liposomes could also be useful in situations where individuals were exposed to toxic substances (e.g., chemical warfare agents) causing skin damage by free radical mechanisms. Inhalation and intratracheal administration can be useful for those situations in which pulmonary tissues are subjected to oxidative stress such as with influenza infection or inhalation of toxic substances such as paraquat (4).

Intravenous administration would primarily be limited to situations in which oxidative stress is a component of an acute trauma or disease. The intravenous use of antioxidant liposomes has the potential for rapidly increasing the plasma and tissue concentration of antioxidants far beyond what oral administration could achieve. Moreover, the proteolytic and bioselective processes of the gastrointestinal tract do not limit the types of antioxidants that can be administered via intravenous antioxidant liposomes. For example, it is known that plasma levels of alpha-tocopherol are about ten times higher than the levels of gamma-tocopherol despite the fact that dietary levels of gamma-tocopherol are at least two times that of alpha-tocopherol. Nevertheless, gamma-tocopherol has a unique chemical ability to detoxify peroxynitrite that is not shared with alpha-tocopherol (43). Peroxynitrite is a powerful oxidant formed by the reaction of nitric oxide with superoxide radicals (see reaction 5) and may be an important mediator of acute oxidant tissue damage. It is reasonable to suspect, therefore, that medical situations could arise in which it would be desirable to rapidly increase plasma (and tissue) levels of gamma-tocopherol. The poor bioavailability of orally administered gamma-tocopherol makes

this very difficult to accomplish. This limitation could, however, be overcome by the intravenous administration of liposomes containing gamma-tocopherol.

Vitamin E used in oral supplements is often in the form of a tocopheryl ester such as tocopheryl acetate or tocopheryl succinate. Tocopheryl esters are not, however, absorbed and must first be acted upon by intestinal esterases to liberate the unesterified tocopherol. It is interesting, therefore, that alpha-tocopheryl succinate but not alpha-tocopherol has been found to inhibit the activation of nuclear factor kappa B (NFkappaB) in cultured macrophages (44). NFkappaB is a key transcription factor that regulates the expression of many inflammatory cytokines. Alpha-tocopheryl succinate can be incorporated into liposomes and intravenous injection would deliver this form of vitamin E to phagocytic cells (45). Oral administration of tocopheryl succinate would not, however, be expected to deliver this form of vitamin E to cells.

It is very significant that Cu,ZnSOD-liposomes administered by intravenous injection can penetrate the blood-brain barrier and significantly elevate brain levels of SOD activity within 24 hours (46,47). Moreover, the intravenous administration of Cu,ZnSOD-liposomes to rats can reduce cerebral infarction caused by ischemia (47) and also inhibit learning dysfunction caused by a low dose of total body irradiation (48). Surprisingly, intraperitoneal injection of SOD-liposomes has also been found to increase the brain levels of SOD in gerbils and to inhibit ischemia/reperfusion oxidative stress (49).

A major problem with conventional liposomes is that they are recognized by the immune system as foreign substances and are rapidly removed from circulation by the phagocytic cells of the reticuloendothelial system. The Kupffer cells of the liver are the most abundant population

of phagocytic cells in the body. In some circumstances, however, the uptake of conventional liposomes by hepatic Kupffer cells can actually be an advantage. Carbon tetrachloride (CCl_4), for example, is known to induce hepatotoxicity by a free radical mediated mechanism. Yao et al. (45) found that intravenous administration of liposomes containing vitamin E (TOH-liposomes) was very effective in decreasing mortality in mice given a lethal dose of CCl_4. The TOH-liposomes were found to primarily accumulate in the Kupffer cells of the liver.

In recent years considerable advances have been made in the design of stealth liposomes that are not well recognized by the immune system and therefore have a much longer half-life in circulation than conventional liposomes. Stealth technology employs liposomes with a polymer coating of polyethylene glycol- phosphatidylethanolamine (PEG-PE). Recently, the preparation of pH-sensitive stealth liposomes has been described (50). These liposomes have a prolonged circulation *in vivo* and destabilize at mildly acidic pH thereby being particularly efficient at delivering a water-soluble compound into a cell's cytoplasm. The use of stealth antioxidant liposomes is very new with an increasing commercial interest in their potential therapeutic applications.

6. Antioxidant Liposomes and Oxidative Stress

Increasing evidence suggests that oxidative stress is an important factor in the aging process and in the etiology of many chronic diseases such as atherosclerosis, ischemic heart disease (51), and cancer (52,53). Schwartz et al. (54) at the Harvard School of Dental Medicine have used the hamster cheek pouch tumor model to explore the potential anticancer use of various antioxidants. This group found that beta-carotene liposomes injected into the oral squamous cell carcinoma of the hamster caused a lysis of the tumor cells but not of normal cells

(54). Retinoids have also been shown to be clinically effective in treating diverse premalignant and malignant conditions such as cutaneous T-cell lymphomas, leukoplakia, squamous cell carcinomas of the skin, and basal cell carcinomas (55,56). Several investigators have documented dramatic improvement in patients with acute promyelocytic leukemia after treatment with all-trans-retinoic acid (57-59). However, the side effects of oral all-trans-retinoic acid therapy are similar to effects seen with vitamin A: headaches, other central nervous system problems, and dryness of mucosal tissues, erythema, and desquamation of skin. When incorporated in liposomes, all-trans-retinoic acid-associated toxicity is markedly reduced whereas anti-tumor properties, i.e., growth inhibition and differentiation induction of all-trans-retinoic acid are maintained or even enhanced (60,61). Phase I and phase II clinical studies found that plasma levels of all-trans-retinoic acid were maintained at high concentrations even after prolonged treatment of patients with all-trans-retinoic acid-liposomes (62). In general, the use of retinoids is safe and induces complete remission in 80 to 90 % of acute promyelocytic leukemia patients. However, chronic oral administration results in reduced plasma levels associated with disease relapse in the majority of patients; this can be circumvented by using all-trans-retinoic acid-liposomes.

Oxidative stress also contributes to the pathology observed in acute medical problems such as heart attack (51,63-66), respiratory distress syndrome (67), trauma (3) , irradiation (48), cold injury (68) and certain types of infectious diseases such as influenza and HIV infection. Evidence suggests that trauma to the brain results in the overproduction of superoxide radicals that may contribute to edema (69,70). Antioxidant liposomes containing SOD have been used

effectively to treat posttraumatic brain edema (69,70) and neurological dysfunctions in rats (71).

Retinopathy of prematurity is a leading cause of blindness in premature and low birth weight infants who are often treated with high levels of oxygen due to surfactant deficiency. Considerable evidence (72) indicates that oxidative stress is a major contributor to this disease. In an animal model, Niesman et al. (73) found that intraperitoneal administration of SOD encapsulated in polyethylene glycol modified liposomes resulted in a significant increase in retinal superoxide dismutase activity and an improved tolerance to high oxygen levels. Despite the enormous health-related significance, there are no clinical trials testing the efficacy of antioxidant liposomes to treat retinopathy of prematurity.

7. Pulmonary Applications of Antioxidant Liposomes

7.1 Potential Clinical Applications:

Premature children often suffer from respiratory distress syndrome because they lack the capacity to synthesize pulmonary surfactant (74). Surfactant is necessary to maintain proper expansion of the small air sacs in the lung. If surfactant levels are low, the small air sacs in the lungs collapse resulting in poor oxygen delivery (hypoxia) to tissues. Infants deficient in surfactant therefore require treatment with high levels of oxygen in order to prevent damage to their vital organs. Unfortunately, premature infants are often deficient in antioxidants that are necessary to protect organs from injury caused by high concentrations of oxygen. The combination of surfactant deficiency and the presence of oxygen free radicals promote the development of chronic lung disease (bronchopulmonary dysplasia or BPD). BPD is a major cause of morbidity and mortality in premature infants. An estimated 50% of all neonatal deaths

result from BPD or its complications. In the adult form of respiratory distress syndrome (ARDS), antioxidants such as N-acetylcysteine are recognized for their role in reducing the duration of acute lung injury (75,76). The rationale for using antioxidant liposomes to treat respiratory distress in premature infants or adults is certainly compelling and supported by the animal models detailed below. However, almost no clinical trials have been initiated.

7.2 Animal models:

Shek et al. (77) have discussed the general application of liposomes for improved drug delivery to pulmonary tissues. These authors point out that the delivery of drugs to the lung via liposomes is particularly useful because it can minimize extrapulmonary side effects and potentially result in increased drug retention time. In addition (as discussed above), liposomes for delivery by inhalation or instillation can encapsulate enzyme and/or chemical substances that cannot be delivered by an oral route. Smith and Anderson (78) demonstrated that intratracheally administered liposomes (with phosphatidyl choline, cholesterol and stearylamine) have a long retention time (more than 5 days) in the mouse lung. Liposomes with entrapped Cu,Zn superoxide dismutase and catalase (Cu,ZnSOD+CAT-liposomes) were intratracheally instilled in rabbits and the alveolar distribution of the antioxidants measured after 4 and 24 hours (79). The results indicate that Cu,ZnSOD+CAT-liposomes could increase both SOD and CAT activities in distal lung cells, including alveolar type I, alveolar type II cells, and macrophages. More recent studies by Walther et al. (80) have shown that intratracheal administration of CuZn-CAT-liposomes to premature rabbits can increase the lung SOD activity and protect against hyperoxic lung injury. Moreover, intratracheal delivery of SOD-liposomes or CAT-liposomes does not

down-regulate mRNA synthesis of these enzymes in the premature rabbit lung (81).

Archer et al. (82) have made effective use of the isolated perfused rat lung to study the role of oxygen radicals in modulating pulmonary vascular tone. This group showed that the generation of oxygen radicals (from xanthine-xanthine oxidase) decreased pulmonary vascular presser response to alveolar hypoxia. Either pretreatment of the lung with desferrioxamine or a mixture of superoxide and catalase liposomes inhibited decreases in pulmonary vascular reactivity. Superoxide dismutase administered free in solution or combined with catalase in liposomes, increased the normoxic pulmonary arterial pressure and enhanced vascular reactivity to angiotensin II and hypoxia (82).

In a rat model, Freeman et al. (83) have shown that intravenous injection of SOD-liposomes or CAT-liposomes can increase (two to four fold) the lung associated specific activity of these antioxidant enzymes and also provide resistance to oxygen injury. Intravenous injection of non-entrapped (i.e., free) SOD or CAT (in the absence or presence of control liposomes) neither increased the specific lung activities of these enzymes nor provided resistance to oxygen toxicity. Similarly, intratracheal administration of SOD-liposomes or CAT-liposomes (negatively charged and multilamellar) to rats resulted in a significant elevation of lung SOD or CAT activity as well as resistance to pulmonary oxygen toxicity (84).

Barnard et al. (85) have demonstrated that instillation of cationic SOD+CAT-liposomes in a rabbit model was effective in preventing the increase in pulmonary filtration coefficient (a sensitive index of microvascular permeability) due to free radical-initiated lung injury. Repair of lung injury was inhibited by inhalation of elevated oxygen concentrations. This is of particular

importance to the preterm human infant who may be exposed to elevated oxygen concentrations for weeks or months that could result in the chronic pneumopathy known as bronchopulmonary dysplasia. Treatment with liposome-encapsulated SOD and catalase conferred protection against the cytotoxic effects of 50% and 95% oxygen (86,87) and also protect against cell death (88).

Briscoe et al. (89) have evaluated the delivery of SOD to cultured fetal rat pulmonary epithelial cells via pH-sensitive liposomes. A five-fold increase in cellular SOD activity was observed after incubating the culture cells with the pH sensitive SOD-liposomes (89). Fetal pulmonary epithelial cells express a high affinity receptor for surfactant protein A (SP-A). This receptor can be used to target liposome delivery to these cells by incorporating SP-A during the preparation of the SOD-liposomes (89,90). The presence of SP-A in the SOD-liposomes facilitates their uptake by pulmonary epithelial cells (89,90).

Considerable evidence suggests that oxidative injury to lung tissues can be mediated by neutrophils (91). Phorbol myristate acetate (PMA) has often been used to induce neutrophil mediated lung injury in animal models. It is significant, therefore, that liposomes (dipalmitoylphosphatidylcholine) with alpha-tocopherol are able to counteract some PMA induced lung injury in a rat model (91). In contrast, rats pretreated with blank liposomes (no alpha-tocopherol) showed no protection from PMA induced lung injury (91).

Paraquat has also been used to induce oxidative lung injuries in animal models (4). Suntres and Shek (4) have compared the ability of alpha-tocopherol liposomes (TOH-liposome) or liposomes with both alpha-tocopherol and glutathione (TOH+GSH-liposome) to inhibit paraquat induced lung damage in a rat model. Lung damage was assessed by increases in lung

weight (caused by edema) and decreases in lung activities of angiotensin converting enzyme (ACE) that reflects damage to endothelial and alveolar type II epithelial cells. These investigators found that both TOH-liposomes and TOH+GSH-liposomes were equally effective in preventing loss of lung ACE activity but that TOH+GSH-liposomes were more effective in preventing injury to alveolar type II epithelial cells (4). Interestingly, neither antioxidant liposome was effective in preventing lung edema (4).

Liposomes encapsulated with catalase (CAT-liposomes) have also been found to be efficacious in preventing chronic pulmonary oxygen toxicity in young rats (92). In this work, rats were treated with 100% oxygen for 8 days and also given daily intratracheal injections of the CAT-liposomes (with 160 U of CAT) that prevented chronic lung toxicity. Liposomes encapsulated with superoxide dismutase (SOD-liposomes) or with lower levels of CAT (50 U or 70U) did not prevent the chronic lung changes. SOD+CAT-liposomes are also effective in protecting lung tissues from bleomycin-induced injury as evidenced by decreased levels of lipid peroxidation products (93).

Acknowledgments

This work was support, in part, by grants from the Department of Defense (DOD;DAMD17-99-9550 to W.L,S, M.S. and S.D.), USDA National Research Initiative Competitive Grants Program (Proposal No. 9600976 to W.L.S.), Natural Source Vitamin E Association (to W.L.S.) and NIH grant S06GM0837 (to S.D.)

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Milton G. Smith, M.D. 10/20/00
The First Mustard Consortium Meeting-Crystal City

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- **DO NOT ASK: "WILL A TERRORIST ATTACK OCCUR IN SOME CITY, SOME WHERE"?**

- **ASK: "*WHEN WILL IT HAPPEN*"?**

- ***ARE WE READY?***

- **DO WE HAVE AN ANTIDOTE FOR A CHEMICAL WEAPONS ATTACK?**

Who are we?

The Mustard Consortium is a public-private partnership that was funded through a one million dollar grant from the Army.

It is composed of:

- Meharry Medical College (the Administrative lead institution),
- University of Michigan
- East Tennessee State University
- Center for Blood Research
- Walter Reed Institute for Biomembrane Research
- Amaox, Ltd.

What do we do?

The *primary* mission of the consortium is to develop an antidote and counter measures for the exposure to mustard gas. **Currently there is no antidote!** Consequently our military ground troops have little protection. Our civilian population, particularly in high density areas have even less, or no protection from such attacks with mustards.

Our secondary mission is the continued development of the antioxidant liposome technology and its applications various diseases.

What are mustards

Mustard "gas", also known as H, yperite, sulfur mustard, Kampfstoff Lost)is a viscous liquid. Its chemical name is: 1,1',thiobis[2,chloroethane]. The molecular formula $C_4H_8Cl_2S$, and formula weight 159.08.

Its Chemical Abstracts Service registry number is 505-60-2.

Documented uses of mustards:

- Morocco-1925
- Ethiopia-1935
- China- 1934: 1944
- Large quantities were prepared by the Allies and Axis during WWII.
- Release of mustards in the Bari harbor (Italy)-1943
- Stockpiles by the US and the Soviet Union throughout the Cold War
- Mustards as a weapon are relatively unsophisticated, but effective in achieving the purpose terrorizing the opposition.
- Iran-Iraq 1980's

What are mustards, and what do they do?

Meyer first synthesized mustard in 1886. When pure, "H" is a colorless and odorless liquid. Agent grade material is typically yellow to dark brown; the odor is variously described as similar to that of burning garlic, or a sweetish odor. At the time of detection of the odor of mustards by the human nose it is all ready very late for the exposed individual. For many of the individuals who are close to the release of the gas, are essentially defenseless regardless of protective equipment (such as gas masks even if they are donned). Mustards are radiomimetics, in that they show similar damage as that of radiation exposure (e.g., from a nuclear accident).

The mechanism of action is unknown. One hypothesis is that they function as an oxidant, thereby causing free radicals to be generated. This hypothesis is further supported by the recent work of the Consortium, in that an antioxidant formulation has been found to confer protection against tissue exposed to mustards. Free radicals (oxidants) are damaging to all types of tissues and cells, since they react with them indiscriminately.

Mustards as the gas or liquid exposed to the skin result in the production of vesicles, sloughing of the skin, mimicking severe burns. Inhalation of the gas produces inflammation, and hemorrhaging. Several hours after the exposure these individuals would be left gasping for air. The lungs eventually fill with fluid, blood and fibrous tissue. Individuals relatively close to the blast are likely to die within weeks. Long-term effects of the inhalation or contact with the gas can cause cancers and progressive pulmonary fibrosis respectively. Both of which can eventually lead to great disability or death over a varying periods of time.

What do we do it for?

If our attempts at finding an antidote for mustard gas exposure are proven to be successful, it would necessitate the development of a national policy for use of the antidotes. The policy would be needed to ensure a uniform understanding of the use of the drug and its availability to populations that are at risk for such terrorist attacks.

The animal and cellular models developed by the consortium will serve as a platform for further development of the antioxidant liposomes. It is conceived that this technology will have a multiplicity of uses as a new therapeutic regimen. In the military population, as well as the civilian population the antioxidant liposome could be used as counter measure to the effects of mustards.

Other uses for the antioxidant liposomes are oxidant mediated diseases, which of course have both used in military and civilian medicine. The antioxidant liposomes (AL) have already been demonstrated to suppress inflammation. An example of it's potential clinical use would be for new on set myocardial infarctions (heart attacks). The anticipated effects are:

1) Decrease further damage to the heart due to ischemia
2) Decrease reperfusion injury
3) Decrease post infarction inflammation
4) Decrease the stickiness of platelets, thereby decreasing the likelihood of another blockage of the coronary arteries
5) Decrease in post myocardial infarction inflammation

IF the above benefits of the AL are realized it would be expected that there would be lessen the hospital, and increase speed of recovery. A more rapid convalescence would expectantly return the patient to work much earlier. This is already a known trend as health care procedures and drugs improve.

Decreasing the number of overall sick days related to an illness has large ramifications to the national health care policy. Twenty percent of all hospital costs and 1% of the Gross Domestic Product in 1993 (http://www.rwjf.org/health/020082s.htm) were attributed to intensive care unit stays. Estimated costs of the daily stay and administration of care in an average intensive care unit is approximately 1500 dollars per day.
There are about one million heart attacks per year. Perhaps eighty percent will survive to proceed to a hospital for care (approx. 800,000). Decreasing the intensive care stay for this one disease by just one day would save approximately **12.6 billion dollars.**

The Technology
The "*antioxidant liposome*" the technology under development would serve as the delivery system for the specific antioxidant formulation. The liposome (lipo-fat; soma-body) encapsulates typically a drug or protein. By doing so, it gives the encapsulated substance properties that are not present with a free drug. For instance the liposome can be altered so that it can be directed to the type of organ that a high concentration of the

drug is desired. This is not possible with a free drug. Also properties such as sustained released, or releasing its contents under certain conditions, are all possible.

These are relevant conditions that are of concern in terms of the preparation of a counter measure to mustards. Ground troops are likely to be exposed to chemical or biological weapons in the field. It would be very advantageous to administer the drug in advance (prophylactic administration). If drug levels are already at therapeutic levels and if the soldier is exposed to the mustards then it is anticipated that there will be less lung and skin damage. Given that the antioxidants are proven to have a similar demonstrated effect in later trials then treatment would be envisioned in three phases: phase one- prophylactic treatment, wherein the drug is already on board; phase two- about the time of known exposure a booster of the antioxidants are given (likely to be the free drug); phase three- at the medical facility continued dosing of the antioxidant liposomes to maintain high tissue concentrations until inflammation has subsided.

Why the consortium arrangement?

The consortium arrangement allows the formation of a group of scientist of differing disciplines to work on similar aspects of one project within in a "semi-autonomous virtual environment".

In examination of the scientific literature of different disciplines it becomes obvious that there is little cross-disciplinary learning. It is because of this, that there is a relatively slow synthesis of new ideas. By bringing different disciplines together to work on a single project our intent was to greatly decrease the time that it typically takes to solve a difficult scientific problem. Also the consortium has started the work with a new technology at the outset.

Advantages of the Consortium:

- Speed of discovery
- Different models can rapidly show inconsistencies or confirmation of results
- Since groups are in constant communication corrections can be made, due to ongoing inter group feedback.
- If necessary one discovery can cause other investigators to change experimental design
- We have started with a coherent theory; therefore we have begun with the same premise; as opposed to differing ones.
- Usually such a divergent group would not have common interest.
- There is no dislocation of any investigator.
- Telephone and the "net" markedly increase our speed of communication, timely face to face meetings

- Minimal redundancy of models

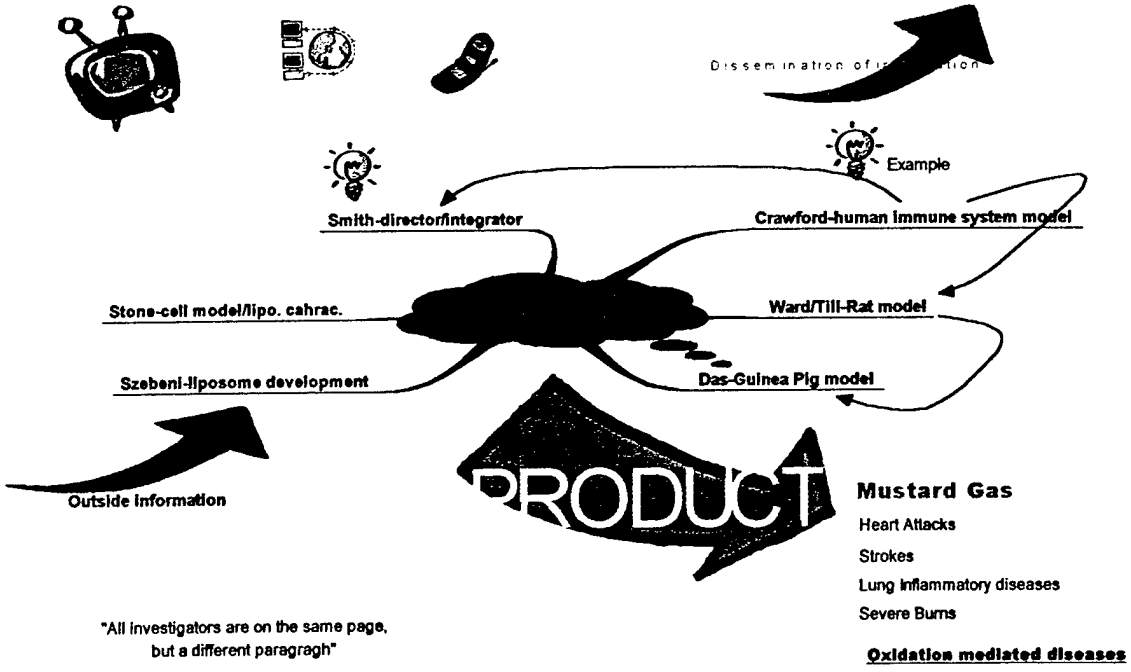
Disadvantages

The structure is uncommon due to the difficulty in arranging such a group.

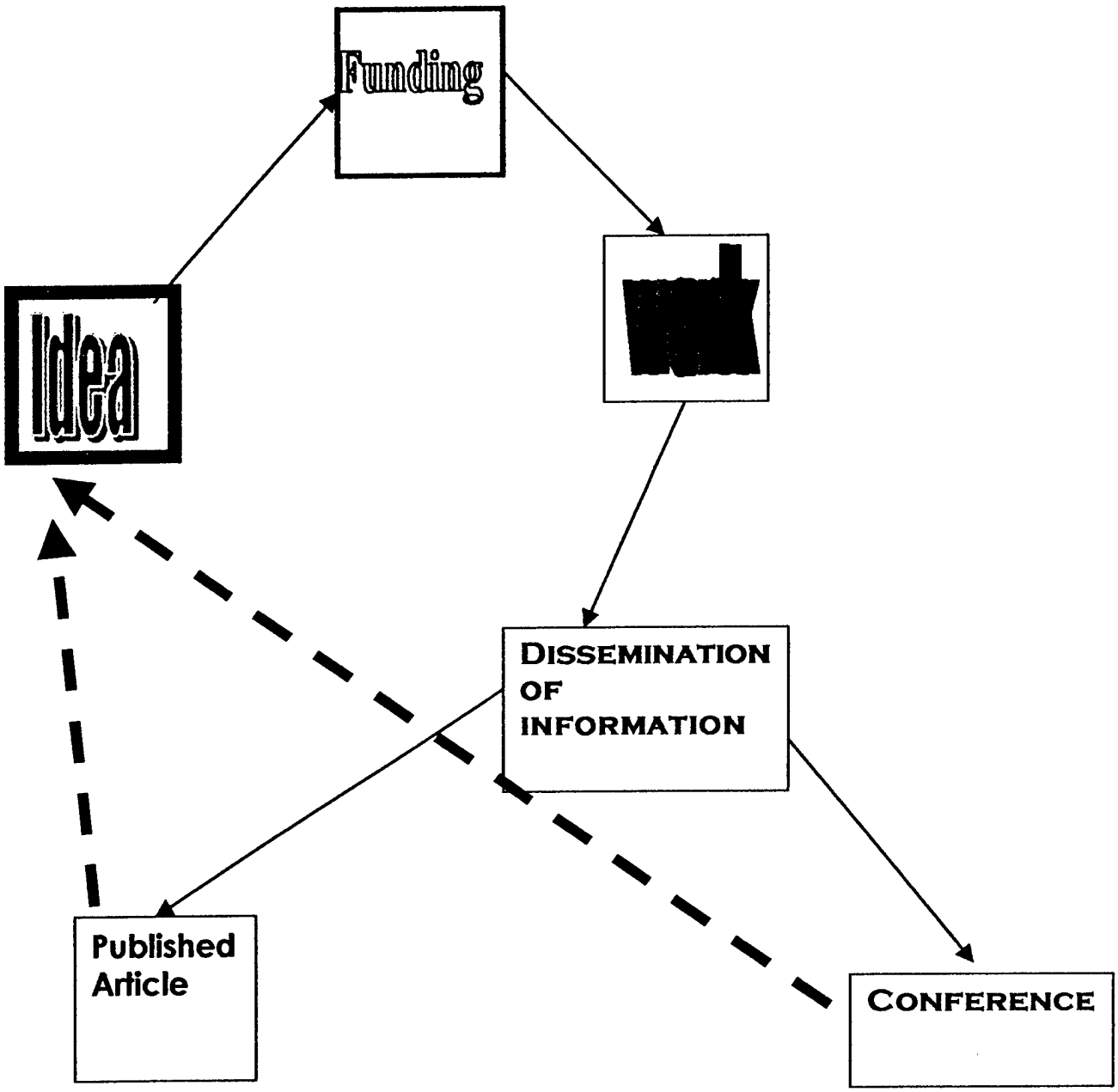
The life cycle of a research project- usual circumstances	**Time lapse**	**The life cycle of a research project in the confines of the Consortium**	Time lapse
1. Idea		Idea	
2. Apply/receive funding		Apply/receive funding	
3. Start work/discovery		Start work/discovery	
4. Dissemination of (intellectual property to public) discovery 5. Feed back on research is limited and stilted (in the form of grant rejection / acceptance, conferences, publications.		Dissemination of information it to primarily the group. Then secondarily to the public information pool (literature). Group can judge the quality of work done by other members. Feed back can occur within a week or two; constant, continuous	
6. May lead to new technology? My not?		New technology already in place! Proof of concept has already taken place!	
7. Or simply another piece of information to be mined in searches by		Component of the overall concept demonstrated to be protective to oxidative damage in a whole	

random investigators through out the world. This process is ongoing		animal and cell line. Usually this done disjointly, no immediate benefit for quick feedback to build to a larger concept. Consortium able to build to the next level concept years	

Conceptual Organization and Work Flow of the Mustard Consortium



Standard Research Organization and Work Flow



Typically a scientific investigator will have an idea, with examples that he/she then attempts to be funded by a given organization. Once the research is underway information is gleaned by the experiments that have been done. The dissemination of the information can occur by a presentation at a conference and/or publishing in books or article (which are usually more current). There is a considerable amount of lag time between obtaining grant money and getting the actual work done. This can be a year or greater. The lag time incurred between getting the funding and the actual publishable work, or presentation is about six to eight months. After the presentation is given members of the audience may act on the information that has been recently learned. This may take an additional six to twelve months.

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